

**THE INTERACTION BETWEEN BACTEROIDES
(CELL SURFACE ANTIGENS) AND THE
IMMUNE SYSTEM IN HEALTH AND DISEASE**

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**"The greatness of a nation and its moral progress
can be judged by the way its animals are treated"**

Gandhi

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ABBREVIATIONS

Abbreviations used frequently and / or unique to this thesis:

Ab (s)	Antibody (ies)
Ag (s)	Antigen (s)
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CBA	Columbia blood agar
CFTB	Complement fixation test buffer
cfu	Colony forming units
CD	Crohn's disease
CP	Capsular polysaccharide
EDDA	Ethylenediamine-N,N'-diacetic acid
EDL	Electron dense layer
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycol-bis (β -aminoethyl ether) N,N,N',N'-tetraacetic acid
HBSS	Hanks' balanced salts solution
HISS	Heat-inactivated sheep serum
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
Ig	Immunoglobulin
KDO	3-deoxy-D-manno-2-octulosonate
LAL	Limulus amoebocyte lysate gelation
LC	Large capsule
LPS (s)	Lipopolysaccharide (s)
Mab (s)	Monoclonal antibody (ies)
MOF	Multiple organ failure
MPRL	Departmental stock culture
NB	Nutrient broth
NC	Non-capsulate
NCTC	National Collection of Type Cultures, UK
OD	Optical density
OMP (s)	Outer membrane protein (s)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCP	Phenol / chloroform / petroleum ether
PF	Pyrogen-free
PPY	Proteose peptone-yeast extract medium
PSA	Polysaccharide A
PSB	Polysaccharide B
R-LPS	Rough-lipopolysaccharide
S-LPS	Smooth-lipopolysaccharide
SAPU	Scottish Antibody Production Unit
SC	Small capsule
SDS	Sodium dodecyl sulphate
SNBTS	Scottish National Blood Transfusion Service
SIRS	Systemic inflammatory response syndrome

TNF	Tumour necrosis factor
UC	Ulcerative colitis
VPI	Virginia Polytechnic Institute, USA
VT	Van Tassell & Wilkins' medium

ABSTRACT

Members of the genus *Bacteroides* are obligately anaerobic Gram-negative rods which constitute a substantial part of the normal colonic microbial flora, and as such play an important part in gut homeostasis and function. When they escape from the gut, they frequently become pathogenic, and *B. fragilis*, the type species, is the principal cause of anaerobic infections in man. Despite this, there are certain diseases where *Bacteroides* species could be very important, but to date have been overlooked or dismissed as insignificant. Sepsis is an example of such a disease; it is widely thought that enterobacteria and their products are exclusively responsible for the development of sepsis, despite the fact that they are greatly outnumbered in the gut by *Bacteroides* species. The aim of this thesis was to examine the interaction between *Bacteroides* species, in particular their cell surface antigens, and selected aspects of the immune system, both in health and disease.

Complement-mediated killing of *Bacteroides* species was examined. It was found that growth environment could have a profound effect on serum sensitivity of certain strains. When grown in proteose peptone-yeast extract medium, all 12 strains tested were sensitive to complement. However, when grown in Van Tassell and Wilkins' medium, six of the strains became markedly more complement-resistant, and when grown in heat-inactivated sheep serum, five of these six strains became totally resistant. Two *B. fragilis* strains were selected which demonstrated these differences, and expression of their surface antigens in the three media was examined to try to discover the biochemical basis for the observed changes in complement resistance. Differences in degree of encapsulation, outer membrane protein expression and lipopolysaccharide (LPS) expression in the three media were seen, and in one of the strains, it appeared that complement resistance was due, at least in part, to the expression of smooth LPS.

Immunoglobulin G (IgG) levels to bacteroides LPS, and to a cocktail of rough LPS from three enterobacteria and *P. aeruginosa* were examined in enzyme-linked immunosorbent assay (ELISA), firstly in healthy individuals (641 blood donors). All donors screened had anti-bacteroides and anti-enterobacterial LPS IgG present in their serum, with levels highly variable between different individuals. The degree of cross-reactivity of the serum IgG was examined by inhibition ELISA. This showed some cross-reactivity between the different anti-bacteroides LPS IgG, but very little between the anti-bacteroides LPS IgG and the anti-enterobacterial LPS IgG. Serum IgG was then measured daily over five to nine day periods in 12 sepsis patients (six survivors, six non-survivors) and in a healthy individual. In all patients IgG levels fluctuated to a greater extent than in a healthy subject. Variations all followed similar trends, and indicated that exposure to bacteroides LPS had occurred. In five of the six survivors, IgG levels increased at the end of the period, whilst in four of the non-survivors levels decreased, with an exception showing increasing levels to *B. fragilis* LPS. In five of the non-survivors, IgG levels against *B. fragilis* LPS were substantially higher than those against the other LPSs. In summary, this study demonstrated that there were some trends in antibody kinetics recognised which suggest that bacteroides LPS may be significant in sepsis.

PUBLICATIONS

Allan E & Poxton IR (1993). The influence of growth medium on serum sensitivity of *Bacteroides*. *J Med Microbiol* **39 Suppl 1**: Abstract 335.

Allan E & Poxton IR (1994). The influence of growth medium on serum sensitivity of *Bacteroides* spp. *J Med Microbiol* **41**: 45-50.

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Allan E, Poxton IR & Barclay GR (1995). Anti-bacteroides lipopolysaccharide IgG levels in healthy adults and sepsis patients. *FEMS Immunol Med Microbiol* **11**: 5-12.

Allan E, Riley S & Poxton IR (1995). Investigation of the mechanism of complement resistance in *Bacteroides fragilis*. *IX International Symposium of the Society for Anaerobic Microbiology, Cambridge*: Abstract 5: 10 (Oral Presentation).

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DECLARATION

All of the experimental procedures in this thesis were carried out by the author unless stated otherwise in the acknowledgements.

CHAPTER 1

INTRODUCTION

The relationships which exist between micro-organisms and their hosts can be broadly placed into three categories:

- **Commensalism:** the micro-organism derives benefit from the host without causing the host any damage.
- **Mutualism / Symbiosis:** both the micro-organism and the host benefit from the relationship.
- **Parasitism:** the micro-organism benefits from the relationship to the detriment of the host.

These descriptions suggest clear-cut categories into which all micro-organisms can be conveniently placed. However, in reality the relationship between micro-organism and host is often much less simplistic; a commensal or symbiotic micro-organism may under certain circumstances become parasitic, or a commensal organism may be to an extent parasitic without causing significant damage to the host.

The human body has an extensive and varied microbial flora which is in a state of dynamic equilibrium. In health, the relationship between micro-organisms and man is either one of commensalism - for example skin-associated bacteria, or mutualistic - for example intestinal bacteria which are provided with both the environment and nutrients necessary for their survival, and in return aid in food degradation and produce vitamins. In disease, the relationship is parasitic, and the greater the capacity a micro-organism has to cause disease, the more pathogenic it is said to be. **Opportunistic pathogens** will only cause disease in immunocompromised hosts. At

the other extreme, **strict pathogens** are always associated with disease. Between these two extremes, **facultative pathogens** (the majority of organisms) will cause disease in a healthy or relatively healthy host if they get out of their usual niche, or if the normal balance of microbial flora is disrupted.

Man has evolved a sophisticated recognition system for potential pathogens, along with highly efficient inflammatory and immune responses to prevent their growth and spread, and to eradicate them from the body. In turn, pathogenic micro-organisms have evolved ways of bypassing these host defences. The ability to adapt, evolve and exploit weaknesses in host defences is one shared by most successful pathogens.

This thesis will examine the interaction between *Bacteroides* spp. (Gram-negative facultative pathogens) and selected aspects of the immune system both in health and disease. First it is necessary to review the general structure of Gram-negative bacteria and the host / pathogen interaction.

1.1 THE GRAM-NEGATIVE CELL ENVELOPE

The cell envelope of a Gram-negative bacterium is a complex structure which gives the cell shape and rigidity, acts as a protective barrier and also as a functional interface through which the bacterium senses and responds to the environment (Poxton 1993). The major structures which constitute the cell envelope are the inner or cytoplasmic membrane, the periplasm and the outer membrane (Figure 1). In addition, many Gram-negative bacteria also possess fimbriae, flagella and a polysaccharide capsule or slime layer.

The areas of the envelope which are in direct contact with the environment outside the cell (including the host immune system in the case of potential pathogens) are the outer membrane and the structures beyond. These are discussed in detail below.

1.1.1 THE OUTER MEMBRANE

The outer membrane is composed of three major components: protein, lipopolysaccharide (LPS) and phospholipid. It acts as a selective permeability barrier, normally only allowing the passage of small hydrophilic molecules through to the periplasm. This barrier function is controlled by proteins and LPS.

1.1.1a Outer Membrane Proteins

There are three types of outer membrane proteins (OMPs): major OMPs or porins, minor OMPs and lipoproteins (Benz 1988). Porins form relatively non-specific diffusion channels or pores in the outer membrane through which small hydrophilic nutrients can pass. Minor OMPs act as specific transport channels for molecules which are at low concentrations in the environment (e.g. iron and vitamins) or are too large to pass through porins. Lipoproteins have no pore function, but help to maintain the structure and rigidity of the outer membrane. The degree of expression of outer membrane proteins is environmentally regulated.

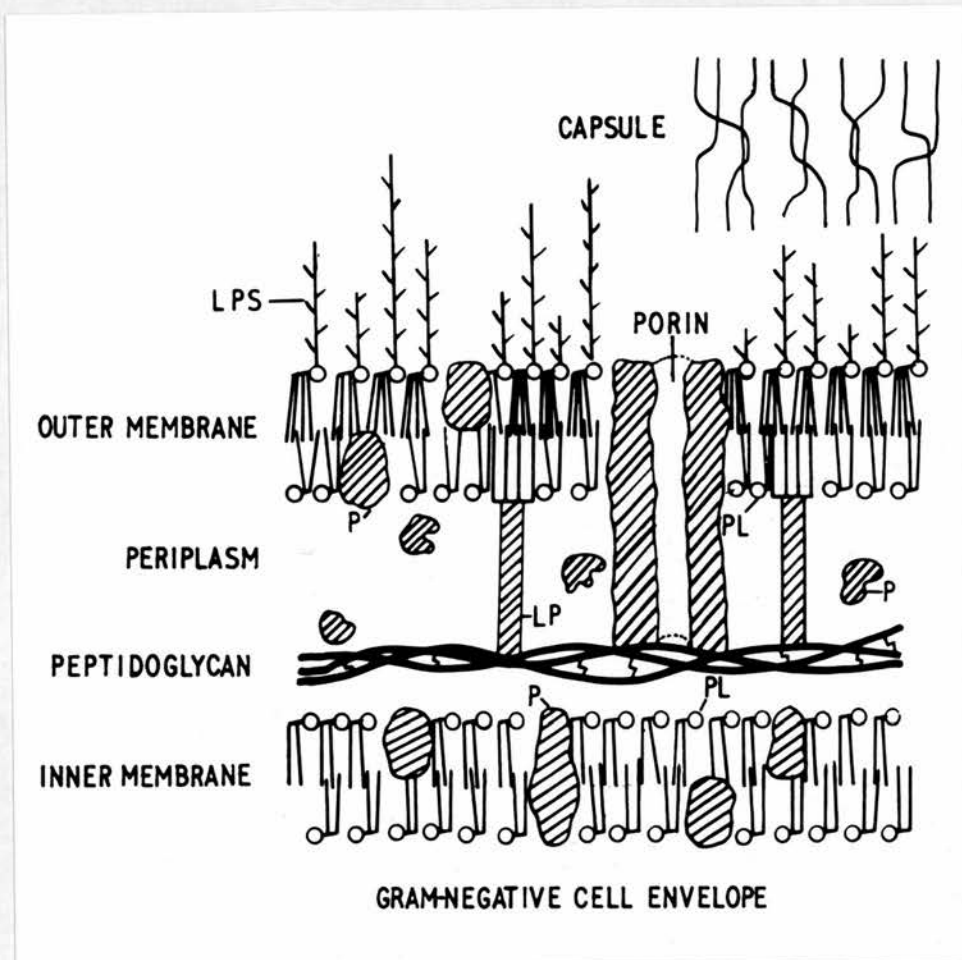


Figure 1. The cell surface of a Gram-negative bacterium.

LP = lipoprotein; P = protein; PL = phospholipid. From Hancock & Poxton (1988).

1.1.1b Lipopolysaccharide

LPS is unique to Gram-negative bacteria. It is found in the outer leaflet of the outer membrane where it replaces phospholipids (Stanier 1986). It is made up of three major components: lipid A, core oligosaccharide and O-polysaccharide or O-antigen.

- **Lipid A:** this region is composed of long-chain saturated fatty acids, and anchors the LPS to the outer membrane. The structure of lipid A results in very close packing of LPS molecules into the membrane, creating an extremely rigid barrier which prevents the passage of hydrophobic molecules into or out of the cell (Poxton 1993). It is the region of LPS responsible for the pathophysiological or "endotoxic" effects of LPS (endotoxin is synonymous with LPS in an infected host: Rietschel & Brade 1992). Lipid A is generally highly conserved between different Gram-negative bacteria (Mutharia *et al* 1984), but substitution patterns of fatty acids and phosphates, and chain lengths of fatty acids are variable which can result in large differences in biological activities (degree of endotoxicity) of different LPSs (Poxton 1993).

- **Core oligosaccharide:** the core oligosaccharide is composed of inner and outer regions; the inner core is fairly conserved between different bacterial species (Jansson *et al* 1981) and contains two unusual sugars - heptose and 3-deoxy-D-manno-2-octulosonate (KDO) which links the core to lipid A via an acid-labile bond (Wicken & Knox 1980).

- **O-antigen:** the O-antigen is composed of a long chain of repeating oligosaccharide units which is highly variable between different bacterial species and strains, and is often an important determinant of pathogenicity (see Section 1.2.2). Not all Gram-negative bacteria possess an O-antigen; those which do are referred to as having smooth (S-) LPS and those which lack it are referred to as having rough (R-) LPS. This can be visualised on polyacrylamide gels, where smooth strains demonstrate a

characteristic "ladder pattern" in the high molecular weight region, with each "rung" of the ladder representing the addition of one repeating oligosaccharide unit.

In order for a Gram-negative bacterium to be viable, it must possess lipid A and KDO; mutants defective in synthesis of these regions have never been isolated (Davis 1990).

1.1.2 CAPSULAR POLYSACCHARIDE AND SLIME LAYERS

Many Gram-negative bacteria are covered by either a discrete polysaccharide capsule or a loosely attached slime layer. These form a hydrophilic matrix around the bacterium which is used for adhesion, aggregation and biofilm formation (Poxton 1993) and is often involved in evasion of the immune system by pathogenic bacteria (see Section 1.2.2).

1.1.3 FIMBRIAE AND FLAGELLA

Most Gram-negative bacteria have short, rigid, filamentous appendages made of protein, called fimbriae (formerly called pili). There are usually several hundred per cell, and they are fairly evenly distributed around the cell. Their function is one of adhesion, and they therefore often play an important role in pathogenesis.

Flagella are present on many rod-shaped and curved bacteria, and confer motility on the cell. They are longer and less rigid than fimbriae, are also made of protein and may be polar (present at one or both poles of a cell) or peritrichous (all around the cell). Flagella are important in pathogenesis when the bacterium is growing in liquid - for example urine (Davis 1990).

1.1.4 ENVIRONMENTAL REGULATION OF THE CELL ENVELOPE

The ability of a bacterium to survive and grow anywhere is entirely dependent on its

ability to adapt to the surrounding environment. In the case of a potential pathogen trying to survive in a host, this will necessitate rapidly finding a place to establish itself and grow, scavenging for nutrients and evading host defence mechanisms. All of these adaptive processes are mediated by the cell envelope, and so the more quickly a bacterium can adapt by appropriately changing its cell surface, the better its chances of survival (Smith 1990). There have been numerous reports of variation in expression of cell surface molecules with environment. Moreover, it has been observed that expression of surface molecules by pathogenic bacteria grown *in vivo* or *in vitro* in physiological media such as serum is markedly different from expression in rich laboratory media (Brown & Williams 1985, Miller *et al* 1989b, Smith 1990, Nelson *et al* 1991, Camprubi *et al* 1992), which is obviously an important consideration when studying the mechanisms of pathogenesis of any given bacterium.

1.2 THE INTERACTION BETWEEN THE HOST AND THE PATHOGEN

Any infectious agent whether a bacterium, fungus, virus or parasite contains a huge variety of proteins and polysaccharides which the host recognises as foreign. These substances, collectively known as antigens (Ags) are responsible for the induction of the host immune response.

This section will focus on the relationship between man and potentially pathogenic bacteria.

1.2.1 HOST DEFENCES

Man has two different defence mechanisms with which to fight off potential invaders. These act in tandem to give maximum protection (Janeway & Travers 1994).

- **Innate immunity.** Responsible for the early phases of the host response to infection. A variety of innate resistance mechanisms recognise and respond to the presence of a pathogen. These are present in all individuals at all times, are non-specific and do not increase with repeated exposure to any given pathogen (i.e. do not develop immunological memory).
- **Acquired immunity.** Directed against specific micro-organisms, and mediated by antigen-specific lymphocytes. This response also includes the development of an immunological memory, which gives long-term protection from the pathogen against which it is directed and gives a quicker and more intense response on a second encounter with the pathogen.

1.2.1a Innate Immunity

Innate immunity can be divided into two categories - external and internal defence mechanisms. This division is not clear-cut as many of the defence mechanisms

usually regarded as being internal also play a role in external defence against infection.

External Defences

The external barriers which help to protect man from potential pathogens are summarised in Figure 2.

Internal Defences - Cellular

Phagocytic cells play a vital role in innate immunity by ingesting and destroying invading organisms. Phagocytic cells in man are comprised of neutrophils, monocytes and macrophages (Janeway & Travers 1994). All of these cell types originate in the bone marrow. Neutrophils and monocytes are found in blood; monocytes differentiate in tissues to become macrophages. All types of phagocyte are attracted to, and engulf antigenic material much more efficiently if the Ag is **opsonised** (made more attractive to the phagocyte) with antibody (Ab) and complement (see Section 1.2.1b).

Internal Defences - Humoral

Many enzymes and proteins contribute to man's innate immune system. They are found in tissues and body fluids such as blood, lymph, saliva, mucus and tears. One of the most important of these is complement.

Complement

The complement system is a cascade of over 20 heat-labile proteins in serum which lyses Gram-negative bacteria and infected cells by forming holes in their membranes. The cascade proceeds by two distinct pathways, which are initiated in different ways.

- **Classical pathway.** The most usual and well-known way for the classical pathway

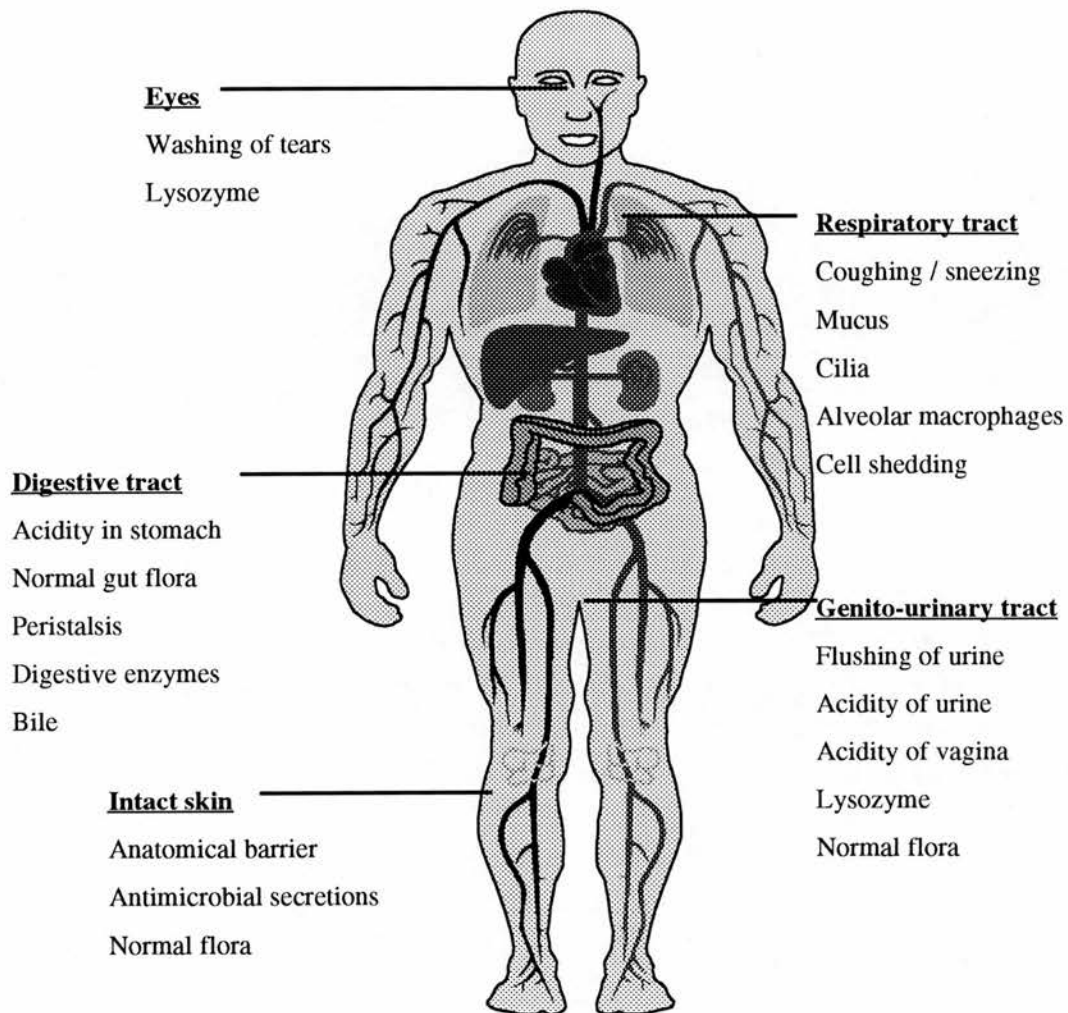


Figure 2. Summary of the external defence mechanisms of the human body.
Adapted from Murray *et al* (1994).

to be initiated is by Ab / Ag complexes (Muller-Eberhard 1975). Only certain classes of Ab are capable of initiating the classical pathway when complexed to Ag (Eisen & Geftter 1990). Other substances have also been found to initiate this pathway, and these include the lipid A region of LPS in certain bacteria (e.g. some *E. coli* spp: Zohair *et al* 1989), porins from Gram-negative bacteria (Sim & Reid 1991), myelin basic protein (Cyang *et al* 1982) and urate crystal surfaces (Giclas *et al* 1979). The first component in this pathway, named C1, binds to Ab / Ag complexes or other initiating substances, becomes activated and then splits proteins C4 and C2 to C4a and C4b, and C2a and C2b respectively (Figure 3). C4b and C2a complex together on the bacterial cell surface to form C3 convertase, which splits protein C3 into C3a and C3b. C3b then complexes with C3 convertase on the membrane to form C5 convertase, which in turn splits C5 into C5a and C5b. C5b binds to the membrane, followed by the sequential addition of proteins C6, C7, C8 and C9. These proteins assemble to form the membrane attack complex which forms a pore in the bacterial cell membrane, which results in lysis of the cell (Kolb *et al* 1972, Poddack & Tschopp 1982).

- **Alternative pathway.** This pathway is initiated by various substances which include the polysaccharide region of LPS (Morrison & Kline 1977), zymosan, teichoic acids, bacteria, some parasites and Ab / Ag complexes where the Ab is not of a class which initiates the classical pathway (Muller-Eberhard 1975). It is constitutively activated at low levels in the blood, thereby providing a constant defence against invading bacteria (Taylor 1983, Abbas *et al* 1991). In the alternative pathway, Factor B binds to C3b, and then with Factor D (Figure 3). This splits the Factor B in the complex to Ba and Bb. Bb remains complexed with the C3b to form C3 convertase. The pathway then proceeds in an analogous fashion to the classical pathway. The classical and alternative pathways merge at the membrane attack complex formation stage, and thereafter are identical.

Complement has other important functions besides cell lysis. Some C3b produced binds directly to the cell membrane rather than forming C5 convertase, and in doing so enhances chemotaxis of phagocytes, which have receptors for C3b. C3a and C5a have powerful anaphylatoxic activity (Frank & Fries 1991). C5a also stimulates phagocytes to release hydrolytic enzymes and to stimulate platelet aggregation, the result of which is thrombosis, swelling and tissue injury (Sims & Wiedner 1991).

Classical Pathway

Alternative Pathway

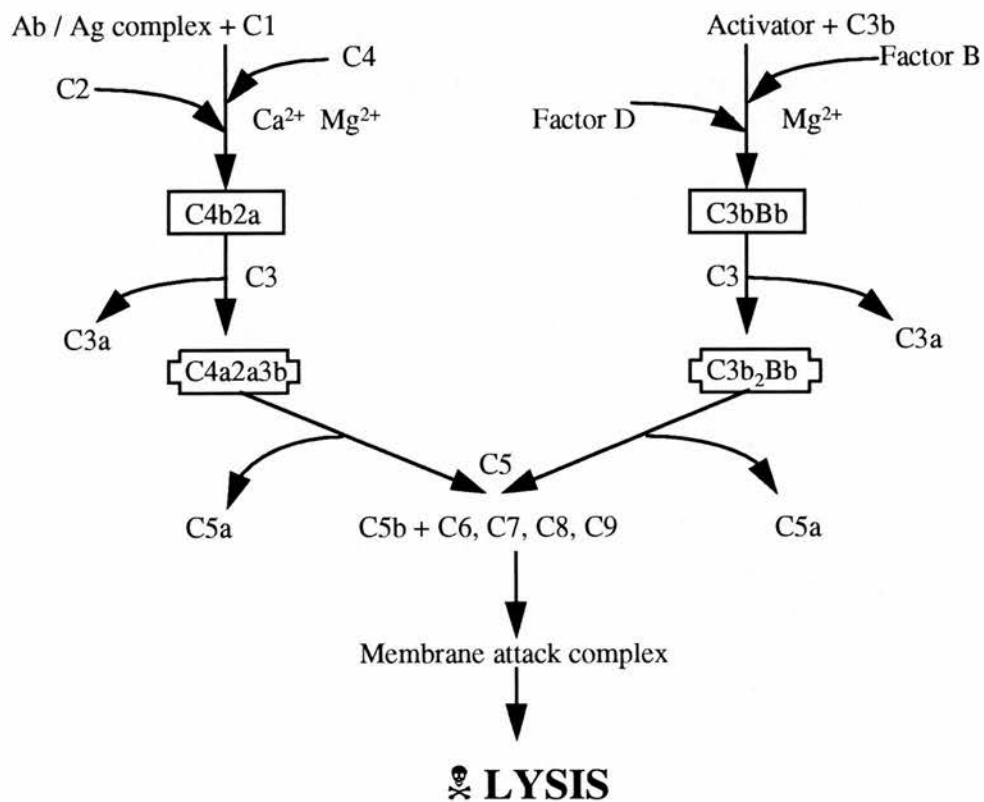


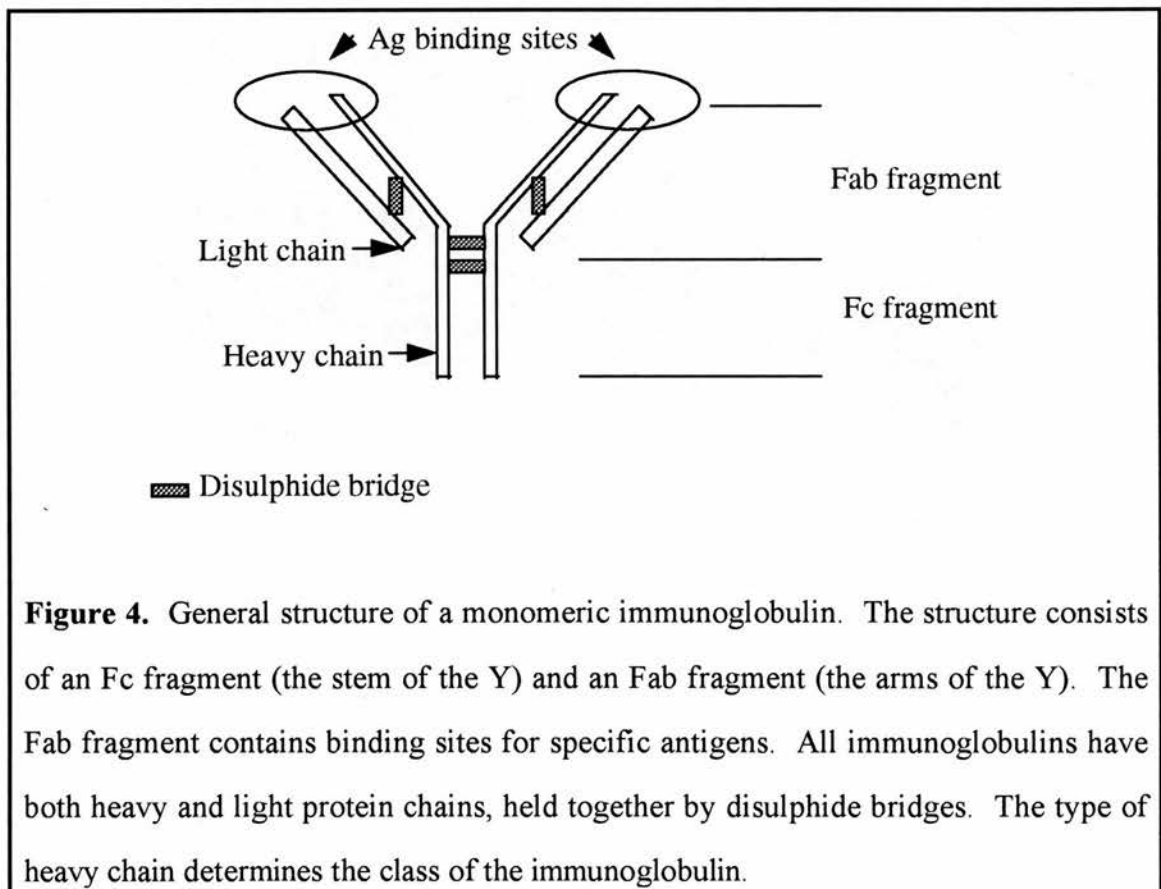
Figure 3. The classical and alternative pathways of complement activation. Adapted from Murray *et al* (1994).

1.2.1b Acquired Immunity

There are two main mechanisms by which the host mounts an acquired immune response against infection - a humoral (antibody) response and a cell-mediated response.

Antibody Response

Antibodies or immunoglobulins (Ig) are proteins in blood and secretions which are produced in response to infection. They are specifically directed against antigens present on the pathogen, and they are the primary agents that protect the body against extracellular bacteria (Sleigh & Timbury 1990). Their basic structure is shown in Figure 4.



Immunoglobulins are produced by B- (bone marrow-derived) lymphocytes. When an Ag encounters B-cells in the spleen or lymph nodes, the B-cells are activated and change into Ig-secreting plasma cells. There are five different classes of Ig: IgM, IgG, IgA, IgE and IgD, which have μ , γ , α , ϵ and δ heavy chains respectively. In addition, IgG and IgA are further divided into subclasses, based on differences in heavy chain structure in the Fc fragments. B-cells produce different classes of Ig at different stages of an infection: initially IgM is produced, and then gene rearrangement occurs within the B-cell and a class switch occurs to one of the other classes.

IgM is produced approximately 5-7 days following infection, and exists in a pentameric form, with the five IgM monomers linked together at the foot of the Fc regions by a J chain (a protein of approximately 15 kDa). The pentamer is very effective at activating the classical complement pathway (Taylor 1983).

IgG is the most abundant Ig in serum and appears approximately 14 days post-infection. There are four subclasses of IgG: IgG1, IgG2 and IgG3 activate the classical complement pathway, and IgG4 activates the alternative pathway. IgG is the only class of Ig to cross the placenta. Memory cells are produced from B-cells as part of the IgG response, and these cells can persist in the blood for decades. On subsequent exposure to the same Ag, these cells will differentiate to form IgG secreting plasma cells within 1-2 days.

IgA is divided into two subclasses - IgA1 and IgA2. Both activate the alternative complement pathway. IgA can exist in monomeric, dimeric or polymeric forms. The IgA dimer has a single J chain (like the IgM pentamer), and also has another chain termed secretory piece, which is necessary for its transport across epithelial cells into external secretions. IgA normally accounts for less than 10% of the total Ig in human

serum, but is the principal Ig in external secretions such as respiratory and intestinal mucin, and as such plays an important role in protecting mucosal surfaces from infection. In human serum, about 80% of the IgA is monomeric, whereas in secretions about 90% is polymeric (mainly dimeric). Memory cells are produced as part of the IgA response.

IgE is mainly found attached to mast cells. These are large cells, occurring principally in submucosal tissues of the gastro-intestinal and respiratory tracts, and in connective tissue along blood vessels, which contain granules of histamine. When Ag binds to the cell-bound IgE, the mast cell releases the histamine granules which induce a localised inflammatory response to clear the infection (discussed below). IgE activates the alternative complement pathway. The function of IgD is unknown (Eisen & Geftter 1990, Janeway & Travers 1994).

The antimicrobial properties of immunoglobulins are as follows (Murray *et al* 1994):

- **Opsonisation** - promote chemotaxis and ingestion by phagocytic cells in conjunction with complement.
- **Block attachment** to host cells (IgA)
- **Neutralise toxins**
- **Agglutinate bacteria**
- **Render motile organisms non-motile**
- **Ab + Ag activates complement.** This results in bacteriolysis and induces an inflammatory response, which brings fresh Ab and phagocytes to the site.

Cell-Mediated Response

T- (thymus-derived) lymphocytes comprise 75% of the circulating lymphocytes in man, and are functionally more diverse than B-cells. Although the binding of Ags to

B-cells is sufficient to initiate Ab production, an optimal B-cell response, and Ig class switching by B-cells, requires additional signals from T-cells which are stimulated by the same Ag. However, not all Ags stimulate T-cells; Those which do not (often bacterial polysaccharides and other high *Mr* Ags) are referred to as T-cell independent. This type of response only results in IgM production, as T-cells are necessary for class switching. Ags which do stimulate T-cells (often proteins) are referred to as T-cell dependent (Eisen & Geftter 1990).

There are different subsets of T-cells: T helper cells respond to specific antigenic stimulation by releasing lymphokines (discussed below). Cytotoxic T-cells destroy infected cells. T suppressor cells are thought to block the activity of B-cells and other T-cells, and are possibly involved in regulation of the immune response.

1.2.1c Cytokines

Cytokines are proteins which are not antigen-specific but which play an important role in modifying the immune response. There are two types of cytokines:

- **Monokines:** produced by cells of the innate immune system (monocytes and macrophages). Include α -interferon, interleukin-1, tumour necrosis factor (TNF) α and colony stimulating factors.
- **Lymphokines:** produced by cells of the acquired immune system (T-lymphocytes). Include γ -interferon, interleukin-2 and TNF β .

The role of cytokines is to modulate the immune response. Some cytokines (e.g. TNF α , interleukin-1, interleukin-6) are proinflammatory, and produce an acute inflammatory response, in order to contain an infection and prevent its spread from the initial focus by stimulating the innate and acquired immune system to respond

more efficiently to Ags. They have the following activities, which together with activation of complement and clotting factors form the definition of an acute inflammatory response (Murray *et al* 1994):

- **Inhibition of macrophage migration** (localises macrophages to the infection site).
- **Chemotactic attraction** of lymphocytes, macrophages and neutrophils to the infected site.
- **Dilatation of capillaries** to increase blood flow to the infected site.
- **Increase in capillary permeability** to allow cellular and humoral components of the immune system increased access to the infected site.
- **Mitogenic activity** or stimulation of unsensitised lymphocytes to divide, and Ig class switching.

Other cytokines (e.g. prostaglandin E₂, interleukin-10) are contra-inflammatory, and act to control the level of, and down-regulate the inflammatory response. These are extremely important, as an uncontrolled or prolonged inflammatory response can have serious, even fatal consequences.

1.2.1d The Normal Flora and the Host Immune Response

Before the subject of host defences is closed, it is important to consider the relationship of the host with the normal microbial flora. The host must be able to distinguish between normal flora and pathogenic bacteria, and only evoke an immune response to invading pathogens or to normal flora when they escape from their usual niche. An area of the body where this is particularly important is the gut. The immune system in the gut mucosa (sometimes referred to as gut-associated lymphoid tissue) must be able to recognise and respond to pathogens in a background of constant stimulation from normal flora- and dietary-derived Ags. The mechanism by which it does this is unknown. It is possible that defects in the gut immune system

may predispose to certain diseases; for example, an under-active mucosal immunity may increase the risk of development of systemic inflammatory response syndrome or sepsis, whilst an over-active mucosal immunity may increase the risk of developing inflammatory bowel disease (Ferguson *et al* 1994). Both of these diseases are discussed in detail in Section 1.5.

1.2.2 THE PATHOGEN FIGHTS BACK

1.2.2a Microbial Virulence

Given the arsenal of host defences, it may appear unlikely that any invading micro-organism would be able to cause disease. However, this is obviously not the case. Bacteria have evolved mechanisms to overcome the host's immune system by adaptation, avoidance or exploitation.

In order to cause disease, a bacterium must be able to colonise host tissues, grow, overcome the immune system and cause damage to the host (Smith 1984). The more able a bacterium is to cause disease, the more pathogenic or virulent it is said to be. Virulence depends on the presence of virulence factors produced by the bacterium. These are either molecules on the surface, or excreted out of the bacterial cell which are able to interact with the host's immune system in various ways to prevent killing (Poxton & Arbuthnott 1990).

The ability of a bacterium to cause disease is dependent on both host and bacterial factors. These are both discussed below.

1.2.2b Host Factors Which May Increase Risk of Disease (Sleigh & Timbury 1990)

- **Malnutrition** - e.g. vitamin or protein deficiency, which will impair the immune system.

- **Age** - the very young (especially pre-term neonates) and the elderly have less efficient immune systems than other people.
- **Impairment of the immune system** by immunosuppressive drugs (e.g. steroids, cytotoxic drugs), or by disease (e.g. leukaemia, AIDS).
- **Change in host's microbial flora** - e.g. after broad spectrum antibiotic treatment harmless bacteria may be depleted, allowing establishment of pathogens.

1.2.2c Bacterial Colonisation

Most bacterial infections are initiated by bacterial attachment to host cells, followed by replication which establishes colonisation (Murray *et al* 1994). Adherence may be mediated by non-specific means, such as polysaccharide capsules, or may require the interaction of structures on the bacterial surface such as lipoteichoic acids, outer membrane proteins, haemagglutinin, flagella or fimbriae with specific glycoprotein or glycolipid receptors on host cells (Beachey 1981). Some examples of bacterial adherence mechanisms are listed below:

- Enterotoxigenic *Escherichia coli* have adhesin molecules on their fimbrial tips which adhere to specific receptors on epithelial cells in the small intestine (Smyth *et al* 1991).
- Most *E. coli* strains that cause pyelonephritis have fimbrial adhesins termed P fimbriae. These binds to specific receptors on urinary tract epithelial cells (Kallenius *et al* 1981).
- *Streptococcus pyogenes* causes throat infections and adheres to fibronectin on buccal epithelial cells, using lipoteichoic acid (Patterson 1991).

1.2.2d Bacterial Growth

Nearly all bacteria that have been appropriately investigated need iron to grow (Guerinot 1994). In the human body, virtually all iron is sequestered in haem-binding

proteins, transferrin and lactoferrin. An important virulence attribute is therefore the ability to obtain iron in this environment. Certain bacteria, such as *E. coli*, secrete siderophores (iron-chelating compounds) into the surrounding environment, and simultaneously increase expression of OMPs which act as receptors for the siderophore / iron complex (Guerinot 1994). Some bacteria, for example *Klebsiella* and *Salmonella* spp. are able to sequester siderophores produced by other bacteria or fungi which they are unable to synthesise themselves (Griffiths 1993). Other bacteria (e.g. *Vibrio cholerae*) produce haemolysins, lyse erythrocytes and obtain the iron within them (Stoebner & Payne 1988). *Neisseria meningitidis*, *N. gonorrhoeae* and *Haemophilus influenzae* utilise iron bound to lactoferrin or transferrin (Guerinot 1994).

1.2.2e Bacterial Resistance to the Immune System

To sustain an infection following initial colonisation and growth, a pathogen must be able to overcome host immunological defence mechanisms. This has been achieved by bacteria in a multitude of ways, the most well-known and important of which are described below.

Capsules and Slime

Capsular polysaccharide and slime impair phagocytosis by their hydrophilic nature (Murray 1994, Poxton & Arbuthnott 1990). They can also prevent phagocytosis by size alone; mucoid *Pseudomonas aeruginosa* grows in microcolonies surrounded by slime (alginate) in the cystic fibrosis lung which are too big to be engulfed (Govan & Glass 1990). Slime can also "mop up" host opsonins such as Ab and complement, thus preventing deposition of the opsonins on the bacterial cells (Murray *et al* 1994). Some bacteria produce capsules which mimic host tissue, with the result that the host's immune system fails to recognise the encapsulated bacterium as foreign (Jann & Jann 1987); for example, Group A streptococci have hyaluronic acid capsules,

which is also present in host connective tissue (Whitnack *et al* 1981). Finally, capsules can mask surface components such as LPS or teichoic acids which would otherwise activate the alternative complement pathway (Marques *et al* 1992).

Antigenic Variation

Certain bacteria can rapidly vary antigenic expression. *Neisseria gonorrhoeae* produces at least 12 different types of fimbriae (Swanson & Berrera 1983, Heckels 1984) with the result that the host is unable to produce sufficient Ab to clear the infection, and vaccines against fimbriae are totally ineffective.

Anti-Immunoglobulin Proteases

Antibodies can be destroyed by specific proteases - e.g. *N. gonorrhoeae* produces an IgA-specific protease (Smith 1984). Some of the effects of Abs are destroyed by interference with complement activation (discussed below).

Prevention of Phagocytic Killing

Certain bacteria can avoid phagocytic killing, either by encapsulation (described above), production of enzymes which lyse phagocytes (e.g. *Clostridium perfringens* α toxin; Poxton & Arbuthnott 1990) or avoidance of intracellular killing (e.g. *Salmonella typhimurium*; Miller *et al* 1989a).

Inhibition of the Action of Complement

The ability to destroy or avoid complement is a major virulence factor in that it will circumvent bacteriolysis and phagocytosis. Some examples of complement resistance are given below.

- Wild-type *Salmonella minnesota* (with smooth LPS) is resistant to complement, whereas rough mutants are sensitive. The membrane attack complex assembles in

smooth strains on the part of the O antigen most distal to the outer membrane and is therefore unable to lyse the cell (Frank *et al* 1987).

- *Moraxella catarrhalis* is a commensal of the upper respiratory tract in children, but some strains can become pathogenic. The clinical isolates are resistant to complement due to a specific OMP which has anti-complement activity; complement-sensitive strains lack this protein, and treatment of complement-resistant strains with trypsin renders them sensitive (Verduin *et al* 1993).

- Group B type III streptococci have sialyated capsules. These mimic host tissue and also mask the teichoic acids on the cell surface which would otherwise initiate the alternative pathway (Marques *et al* 1992).

- Some strains of *Serratia marcescens* secrete a non-specific protease which cleaves amongst other things complement components (Molla *et al* 1989).

- *Mycobacterium leprae* has receptors for complement on its surface, which it uses to facilitate its own phagocytosis, as it is an obligate intracellular pathogen (Schlesinger & Horwitz 1991).

1.2.2f Damage to the Host

The final requirement for pathogenicity - damage to the host - is mediated by extracellular enzymes or other toxic substances produced by the bacterium, or bacterial Ags which either cause damage directly or induce immunopathological reactions:

Extracellular Enzymes

These aid the growth and spread of bacteria by breaking down host tissue and

enabling the bacteria to withstand host defences. For example, *Staphylococcus aureus* produces hyaluronidase which breaks down host tissue, and coagulase which initiates blood clotting, thereby forming a barrier between the bacterium and the immune system components (Iandolo 1989).

Exotoxins

These are proteins exported out of intact bacterial cells which are specific in their actions. They may act only at the infected site or may spread via the blood stream or the nervous system to act systemically. Examples of exotoxins include tetanus toxin, produced by *Clostridium tetani*, which is a neurotoxin, and various enterotoxins produced by *E. coli* which cause watery diarrhoea and may cause haemorrhagic colitis (Poxton & Arbuthnott 1990).

Endotoxin (Lipopolysaccharide)

The toxic part of LPS is in the lipid A region. This part alone can cause all the toxic effects of endotoxin. However, the core and the O-antigen affect the fate of endotoxin in the host; S-LPS is more stable in the host but causes less severe damage than R-LPS. In man, the main biological effects of LPS are pyrexia, complement activation, activation of clotting factors, B-cell mitogenicity and cytokine induction (Poxton & Arbuthnott 1990). If small amounts of LPS are present in the host, the biological effects will be localised and the LPS will be neutralised and cleared. However, if large amounts are present, a systemic inflammatory response can occur which frequently results in death (Bone 1993; discussed in detail in Section 1.5).

Immunopathological Mechanisms

There are four types of immunopathological mechanisms by which bacteria can damage the host besides the effects of endotoxin (Smith 1984). These effects can in some cases be severe or even fatal.

- **Type I - immediate hypersensitivity (anaphylaxis).** IgE bound to mast cells reacts with antigens which would be innocuous in most people. The mast cells release histamines which induce an acute inflammatory response. This is the typical "allergic reaction" seen in hay fever, sensitivity of skin to chemicals etc. In severe cases a systemic reaction can occur, leading to anaphylactic shock and death if not immediately treated.

- **Type II - cytotoxic reaction.** This occurs when bacterial Ags are similar to molecules in host cells; the Ags evoke Abs, which then also react with the host cells - an autoimmune effect. This happens in rheumatic heart fever following streptococcal infection.

- **Type III - Arthus type reactions.** Ab / Ag complexes are deposited on tissue, which are then attacked by phagocytes causing tissue damage. This happens in the kidneys following infection by *Proteus mirabilis*.

- **Type IV - delayed hypersensitivity.** Normally an infected area becomes acutely inflamed, and the infection is cleared. However, if the infection persists for any length of time, host tissue as well as the pathogen will be damaged by phagocytic enzymes. This occurs in tuberculosis (Smith 1994) and cystic fibrosis (Govan & Glass 1990).

1.2.3 SUMMARY

Pathogenicity is multi-factorial and dependent on host and parasite factors. The host-parasite relationship is complex and delicately balanced; host defences must protect against uncontrolled replication of the normal flora and against invasion, but at the same time allow a symbiosis with the normal flora. When the internal balance of the normal flora is disrupted, or when organisms invade from outside, disease results.

1.3 BACTEROIDES IN HEALTH AND DISEASE

Members of the genus *Bacteroides* are Gram-negative obligate anaerobes which make up a major part of the harmless human microbial flora of the gastro-intestinal tract but can become pathogenic under certain circumstances. This section will discuss the taxonomy and classification of *Bacteroides* spp., where they are found in health, the circumstances in which they become pathogenic and the various diseases that they cause.

1.3.1 THE GENUS *BACTEROIDES*: TAXONOMY AND CLASSIFICATION

Until recently, the genus *Bacteroides* consisted of over 50 species, some of which were only very distantly related. This was a result of poor taxonomic classification, which allowed species that only shared marginal biochemical similarities and with a huge range of DNA base composition (guanine + cytosine 28-61 mol %) to be deposited into the genus *Bacteroides* (Shah & Gharbia 1991). Therefore a major reclassification was proposed by Shah & Collins in 1989 to restrict the genus to those species which were closely related chemically and biochemically to the type species *Bacteroides fragilis*. This reduced the number of species in the genus to the following ten, which were formerly known as the "true *Bacteroides*" or the "*B. fragilis* group": *B. fragilis*, *B. caccae*, *B. distasonis*, *B. eggerthii*, *B. merdae*, *B. ovatus*, *B. stercoris*, *B. thetaiotaomicron*, *B. uniformis* and *B. vulgatus*.

Members of the genus *Bacteroides* as it now stands have the following properties (Shah & Collins 1989, Shah & Gharbia 1991):

- Gram-negative, obligately anaerobic, non-spore-forming bacilli or coccobacilli
- Non-motile
- Bile tolerant - grow in, and often stimulated by the presence of 20% bile
- DNA base composition within the range guanine + cytosine 40-48 mol %

- Saccharolytic - produce acetic and succinic acids as the major end products of glucose metabolism
- Possess malate, glutamate, glucose-6-phosphate and 6-phosphogluconate dehydrogenases
- Possess sphingolipids
- Possess predominantly methyl branched long chain fatty acids
- Possess menaquinones as sole respiratory quinones
- Peptidoglycan contains *meso*-diaminopimelic acid

In addition to the ten species currently classified as *Bacteroides*, it has been recently proposed that an eleventh species, *B. variabilis*, be added to the genus as it also possesses the above criteria (R. Brown, personal communication; paper submitted). For the purposes of this thesis, *B. variabilis* is included as a member of the genus *Bacteroides*.

1.3.2 BACTEROIDES IN HEALTH

Members of the genus *Bacteroides* form part of the normal human microbial flora in the large intestine. Human faeces contains approximately 10^{10} - 10^{11} bacteria per gram, with strict anaerobes outnumbering facultative bacteria (such as *E. coli*) by between 100-1000 to 1 (Hentges 1993). *Bacteroides* is the predominant genus in faeces, accounting for approximately 20-30% of all faecal anaerobes isolated (Namavar *et al* 1989). However, the proportion of these organisms relative to other faecal organisms varies greatly with diet; people taking a typical "Western diet" high in saturated fat and low in fibre have a higher proportion of *Bacteroides* spp. than people eating a vegetarian diet. This is thought to be because bile production is stimulated by food with a high fat content, allowing increased growth of *Bacteroides* spp. which are resistant to, or stimulated by the presence of bile (Drasar & Duerden 1991, Thompson *et al* 1992). It has also been suggested previously that emotional

stress may alter the composition of faecal flora (Holdeman *et al* 1976).

1.3.3 RELATIVE FREQUENCIES OF DIFFERENT *BACTEROIDES* SPP. IN THE LARGE INTESTINE

Extensive studies carried out on faecal samples showed *B. vulgatus*, *B. thetaiotaomicron* and *B. distasonis* to be the most common *Bacteroides* spp. present. *B. fragilis* was the least common, accounting for only 0.3-0.6% of all *Bacteroides* spp. isolated (Moore & Holdeman 1974, Holdeman 1976). These and many other similar studies carried out on faecal samples assumed that faecal flora was representative of the flora in the entire colon. A more recent study compared the frequency of *Bacteroides* spp. in faeces, colon lavage fluid and colonic mucosa (Namavar *et al* 1989). This study found *B. vulgatus*, *B. uniformis* and *B. thetaiotaomicron* to be the most common *Bacteroides* spp. in faeces, accounting for approximately 45, 22 and 15% of isolates respectively, and *B. fragilis* to be one of the least common, representing approximately 4% of isolates. However, the frequency of *B. fragilis* increased considerably in colon lavage fluid, coming second only to *B. vulgatus*, and in colonic mucosa *B. fragilis* was the most frequently isolated spp., accounting for approximately 43% of all *Bacteroides* isolates, with *B. vulgatus* accounting for about 25%. *B. fragilis* was the only sp. with a lower prevalence in faeces than colonic mucosa, which suggests that it has a closer association with the gut mucosa than other *Bacteroides* spp., and presumably means that it has a greater capacity to adhere to gut epithelial cells. Moreover, these results suggest that the occurrence of *B. fragilis* in the intestinal flora has previously been underestimated.

1.3.4 NORMAL MICROBIAL FLORA: ADVANTAGES AND DISADVANTAGES

The normal microbial flora can be both advantageous and disadvantageous to the host. One of the principal advantages is that it prevents colonisation by potential

pathogens; gut bacteria release a number of factors with bactericidal activity (e.g. bacteriocins, colicins, metabolic waste products), which together with lack of available oxygen prevent establishment of other species. The sheer number of bacteria present in the normal intestinal flora means that almost all of the available ecological niches are occupied, preventing growth of invading species. In addition, gut bacteria release organic acids, B vitamins and vitamin K which can then be utilised by the host. They are also thought to play an important part in normal development of the immune system; germ-free animals are very vulnerable to, and very poor at coping with infection (Mims *et al* 1993).

The normal flora become disadvantageous when they get out of their usual niche and spread to previously sterile parts of the body, where they can become pathogenic. This usually occurs with gut bacteria following intestinal perforation. *Bacteroides* spp. in the gut are generally thought of as harmless, but when they escape from the gut into other areas of the body they frequently become pathogenic, and are one of the major causes of anaerobic infections in man (Finegold 1995).

1.3.5 ANAEROBIC INFECTIONS: GENERAL OVERVIEW

Anaerobic infections are common, can occur anywhere in the body, are often serious and sometimes fatal. The incidence of anaerobic infections is probably generally underestimated due to difficulties in specimen collection, transportation and processing. Moreover, treatment of these infections often proves difficult, as many anaerobes are inherently resistant to a large range of antibiotics, and failure to treat for anaerobes in mixed infections (which occurs if the anaerobe is not detected) often leads to little or no response (Finegold 1995).

Many anaerobic infections in man are caused by the normal microbial flora, originating mainly from mucosal surfaces and (much less frequently) the skin. Some

of the factors which may predispose a person to anaerobic infection are as follows (Drasar & Duerden 1991):

- Impaired blood supply
- Disruption of epithelial barriers
- Tissue injury (reduces redox potential)
- Compromised host defences
- Presence of aerobic and facultative bacteria (reduces redox potential)
- Bacterial virulence factors

1.3.6 BACTEROIDES IN DISEASE

Bacteroides spp. are involved in a variety of infections including intra-abdominal, pelvic, brain and lung abscesses, and (less frequently) diabetic foot ulcers, wound infections, bacteraemia and endocarditis (Patrick 1993, Finegold 1995). *B. fragilis* is the most commonly isolated anaerobe in clinical specimens, and the most common cause of anaerobic bacteraemia (Lindberg *et al* 1979). Its greater pathogenicity compared to the other *Bacteroides* spp. is thought to be due at least in part to its closer association with the gut mucosa (Namavar *et al* 1989). Other *Bacteroides* spp. are also isolated from infections, but less frequently than *B. fragilis*. In a study by Brook (1989), clinical isolates from abdominal and pelvic wound infections, abscesses and bacteraemia were collected over a 12 year period. *B. fragilis* was the most commonly isolated of the *Bacteroides* spp., accounting for 78% of all isolates, followed by *B. thetaiotaomicron* (14%), *B. ovatus* and *B. vulgatus* (both 3%). Interestingly, although *B. fragilis* is by far the most common clinical isolate, it has been reported that mortality rates are higher from infections involving *B. thetaiotaomicron*, *B. vulgatus* and *B. distasonis* (Chow & Guze 1984, Brook 1989). However, this should be viewed with caution due to the small numbers of patients involved in these studies and the lack of information on patient histories.

By far the most common site from which clinical *Bacteroides* isolates are recovered is intra-abdominal abscesses. These are discussed below.

1.3.7 INTRA-ABDOMINAL ABSCESSSES

1.3.7a Aetiology and Pathology

An abscess consists of a collection of leukocytes (live and dead), bacteria and cell debris surrounded by a thick wall of damaged and inflamed tissue which the host makes to try to contain the infection. It can have serious consequences for the host as it can spread to nearby sites with resultant necrosis of adjacent tissue, acts as a reservoir of bacteria which can then enter the blood stream, and the resulting bacteraemia can cause shock or produce metastatic infections at distant sites (Tally 1993). Intra-abdominal abscesses usually occur as a result of perforation of the gut wall, causing spillage of intestinal contents into the peritoneal cavity. Gut perforation may occur following direct trauma, appendix or diverticular rupture, and is also one of the most frequent post-operative complications following abdominal surgery (Brook 1993).

The infectious process leading to abscess formation is biphasic; it starts with acute inflammation, which will be followed by abscess formation if host defences are unable to eradicate the infection. Both the acute inflammation and the abscess stages of infection are polymicrobial in nature, consisting of a mixture of facultative anaerobes and anaerobic bacteria. At the moment when the colonic contents are spilled, the peritoneal cavity is well-oxygenated. Therefore in the inflammation (peritonitis) stage, facultative anaerobes (mainly *E. coli*) are numerically predominant in the infection. However, strict anaerobes that are able to resist the toxic effects of oxygen remain viable, and when the area becomes more anaerobic (due to facultative bacteria depleting available oxygen and a decrease in blood supply to the site) the anaerobes will grow, and become numerically predominant in the abscess stage

(Finlay-Jones *et al* 1991, Tally 1993).

It is usual in an abscess to have several anaerobic species present. The most frequently isolated anaerobe is *B. fragilis*, followed by *B. thetaiotaomicron*, then other *Bacteroides* spp. (Finegold 1995). Members of the genus *Bacteroides* are generally oxygen-resistant (especially *B. fragilis*), which together with their high numbers in the colon probably explains why they feature so strongly in intra-abdominal abscesses. *Bacteroides* are often numerically predominant, but there may also be other species present such as peptostreptococci, fusobacteria and clostridia (Brook 1993).

1.3.7b Microbial Synergy

For a long time it was thought that the contribution of anaerobes to the virulence of mixed infections was insignificant (Rotstein 1993). However, it was subsequently observed that in experimental models of intra-abdominal infection, abscess formation and lethality were greater when a mixture of a facultative anaerobe and an anaerobe was injected, as opposed to just one or the other (Onderdonk *et al* 1976, Rotstein *et al* 1989). Moreover, it was observed that patients with abscesses who were treated with antibiotics effective against both facultative anaerobes and anaerobes made significantly better recoveries than patients treated with antibiotics effective against facultative anaerobes only (Berne *et al* 1982). It was also shown that killing of facultative anaerobes by phagocytes *in vitro* was inhibited by the presence of *B. fragilis* (Ingham *et al* 1977, Ingham *et al* 1981), and both the capsule (Reid & Patrick 1984) and the LPS (Jones & Gemmell 1986) of *B. fragilis* were implicated in this process. The exact mechanism by which *B. fragilis* impairs phagocytic killing is unclear, but it has become evident that anaerobes and facultative anaerobes exist in a synergistic relationship in a mixed infection. Anaerobes appear to be able to impair killing of both themselves and the facultative bacteria, whilst the facultative bacteria

keep the redox potential low, allowing survival and growth of the anaerobes.

1.3.8 TREATMENT OF INTRA-ABDOMINAL ABSCESES

Successful treatment of intra-abdominal abscesses often proves difficult. A combined medical and surgical approach is usually required; the surgical side involves repair of the perforation which is allowing spillage of intestinal contents into the peritoneal cavity, and drainage of the abscess. However, even after drainage abscesses can often recur. The medical treatment involves antibiotic administration. As mentioned earlier, this is only usually successful if antibiotics are given which will be effective against all of the bacteria in the abscess. Unfortunately there are two major problems with this - firstly a lot of anaerobes are resistant to many antibiotics. *Bacteroides* spp. all contain β lactamases and are therefore resistant to penicillin-based drugs unless the drug also contains a β lactamase inhibitor. They are also resistant to many cephalosporins, and some recent surveys from the USA have reported isolation of clinical *Bacteroides* strains resistant to all antibiotics except the chloramphenicols (Finegold 1995). The second major problem with antibiotic treatment of abscesses is that the nature of the abscess itself reduces the efficacy of many antibiotics; the poor blood supply, low pH, low oxygen content and high bacterial inoculum all impair antibiotic action (Drasar & Duerden 1991). Despite all this, it is usually possible to successfully treat an abscess if a proper diagnosis is made, followed by surgical intervention and administration of an appropriate and large enough course of antibiotics.

1.4 BACTEROIDES VIRULENCE FACTORS

To date, the vast majority of work to determine the virulence mechanisms of *Bacteroides* spp. has been carried out on *B. fragilis*, as it is the most common anaerobic pathogen. This chapter will therefore mainly deal with *B. fragilis*, but other *Bacteroides* spp. will be mentioned where information is available.

Possible virulence determinants of *B. fragilis* include surface polysaccharides (capsular polysaccharide [CP] and LPS), resistance to complement and phagocytosis, nutrient uptake *in vivo*, release of extracellular degradative enzymes, fimbriae and enterotoxin production (Namavar *et al* 1991, Patrick 1993). These are discussed in turn below.

1.4.1 SURFACE POLYSACCHARIDES

There has been a lot of research carried out on the surface polysaccharides of *B. fragilis*, but unfortunately many of the results obtained to date are confusing, controversial and remain open to debate. One of the main reasons for this is that the surface polysaccharides of *B. fragilis* (and probably other *Bacteroides* spp.) are very closely linked and therefore very difficult to purify. This has led to numerous situations in the past of researchers working with material which they have claimed to be "purified" CP or LPS, whereas in fact the material was not pure, and what it actually contained was dependent on the extraction procedure employed to obtain it. Bearing this in mind, there follows a summary of what is known about the CP and LPS of *Bacteroides* spp.

1.4.1a Bacteroides Capsular Polysaccharide

The CP of *B. fragilis* has been considered for many years to be a major determinant of pathogenicity. It was originally reported that *B. fragilis* was the only member of the genus *Bacteroides* to possess a capsule, and that this was the reason why it was

more pathogenic than the other spp. (Kasper 1976, Kasper *et al* 1977, Onderdonk *et al* 1977a). However subsequent studies using light and electron microscopy showed other strains (*B. vulgatus*, *B. thetaiotaomicron* and *B. ovatus*) to have capsules also (Babb & Cummings 1978, Bjornson *et al* 1983). The main areas of research carried out on *Bacteroides* CP are in antigenic variation, virulence of encapsulated spp. and CP structure.

Antigenic Variation of Capsular Polysaccharide

Babb & Cummins (1978) noted varying degrees of encapsulation of different cells within any given strain. Following on from this observation, Patrick and colleagues found that altering the growth medium from nutrient-rich to the minimal medium of Van Tassell & Wilkins resulted in greatly enlarged capsules in *B. fragilis*, facilitating classification and quantification of different cell types (Patrick 1993). The different cell types were separated using Percoll discontinuous density centrifugation; a step gradient of Percoll was produced by layering equal volumes of 80, 60, 40 and 20% Percoll into a test tube. A sample of the test bacterial culture was layered on top of the gradient, and following centrifugation, three distinct sub-populations of cells resulted, which were visualised by light microscopy and India ink staining. Cells at the 0-20% Percoll interface had large capsules (LC), those at the 20-40% interface had small capsules (SC), those at the 40-60% interface were a mixture of SC and non-capsulate (NC) cells and those at the 60-80% interface were NC (Patrick & Reid 1983). Subsequent studies using electron microscopy revealed large and small fibrous networks in the LC and SC populations respectively, and a narrow electron dense layer (EDL) outside the outer membrane in NC cells (Patrick *et al* 1986).

Monoclonal antibodies (MAbs) were produced against the different cell populations, and studies using these gave several surprising revelations. First, the SC was antigenically distinct from the LC, and not simply a smaller amount of LC; second,

the EDL was antigenically distinct from the SC and not simply a smaller amount of fibrous material (Reid *et al* 1987); third, some MABs reacted with both the LC and EDL cell populations - in the LC population the MABs reacted with both cell-associated material and also cell-free material, whereas in the EDL population they reacted with cell-free material only (Lutton *et al* 1991).

This antigenic variation of cell surface structures could be extremely important in the pathogenesis of *B. fragilis* - for example in evasion of the acquired immune system. Unfortunately it has not been taken into consideration by many researchers, with the consequence that many experiments have been carried out using heterogeneous cell populations which have given conflicting and confusing results.

Virulence of Encapsulated *Bacteroides* species

Onderdonk *et al* (1977a) compared the ability of encapsulated and non-encapsulated *B. fragilis* strains to produce abscesses in rats. They found that live encapsulated strains produced abscesses in nearly all cases, whereas live non-encapsulated strains rarely did. In addition, they found that heat-killed encapsulated strains and purified CP produced sterile abscesses in most cases. It was subsequently shown that abscess induction by *B. fragilis* CP could be prevented by prior immunisation with this polysaccharide and that the immune response which protected against abscess formation was T-cell dependent (Onderdonk *et al* 1982, Shapiro *et al* 1982). Whether it was CP that was solely responsible for abscess formation is a matter of debate, as the "purified" CP was probably contaminated with protein and LPS. CP subsequently obtained using improved purification methods (Pantosti *et al* 1991) is still reported to induce abscesses in experimental animals (Tzianabos *et al* 1993), but whether the material is totally pure remains debatable. However it does appear that CP plays a major, if not necessarily exclusive role in abscess formation.

Encapsulated *B. fragilis* strains were reported to adhere to rat epithelial cells better than other *Bacteroides* spp. (which at the time were thought to be non-capsulate: Onderdonk *et al* 1977b). Unfortunately the ability of non-encapsulated *B. fragilis* strains to adhere was not investigated, but despite this, these results are in agreement with the later study by Namavar *et al* (1989) which showed *B. fragilis* to be in closer association with the gut mucosa than other *Bacteroides* spp. The greater capacity of *B. fragilis* to adhere to host cells compared to other *Bacteroides* spp. is likely to be one of the reasons for its greater pathogenicity.

The ability of encapsulated and non-encapsulated *B. fragilis* cells to resist phagocytosis has been investigated (Reid & Patrick 1984). It was found that encapsulated organisms were significantly more resistant to engulfment and killing by phagocytes than their non-capsulate counterparts. This might suggest that in mixed infections capsulate *Bacteroides* spp. prevent phagocytosis of both themselves and facultative anaerobes, possibly by mopping up host opsonins. A study by Brook *et al* (1992), which found 78% of *Bacteroides* spp. isolated from human abscesses to be capsulate, appears to support this theory. However, studies carried out on experimental animals found that *Bacteroides* spp. present in abscesses or implanted chambers were often non-capsulate (Patrick *et al* 1986, Jotwani *et al* 1992). The CP in at least one of these studies (Patrick *et al* 1986) was present as cell-free slime, which would still inhibit phagocytosis. Differences such as these between animal models and human studies highlight the fact that *in vivo* models are often not an accurate representation of what happens in "real life".

Structure of *Bacteroides fragilis* Capsular Polysaccharide

Recent studies have reported that the CP of the type strain *B. fragilis* NCTC 9343 consists of two distinct high molecular weight polysaccharides, termed PSA and PSB (Pantosti *et al* 1991). Each polysaccharide is composed of repeating oligosaccharide

units which both contain positive (amino) and negative (phosphate and / or carboxyl) groups (Baumann *et al* 1992). The overall charge of PSA is neutral, whereas the overall charge of PSB is negative, and the two polysaccharides form a complex structure which is held together by ionic interactions (Tzianabos *et al* 1992).

The structures of hundreds of bacterial polysaccharides are now known, and the presence of oppositely charged groups on the constituent oligosaccharides is highly unusual. Tzianabos *et al* (1992) noted that only polysaccharides with these unusual structures were capable of inducing abscesses in experimental animals. To try to discover which part of the structure was important in abscess formation, chemical modifications were introduced into *B. fragilis* NCTC 9343 CP, and the ability of the modified CP structures to induce abscesses was assessed (Tzianabos *et al* 1994). It was found that the positively and negatively charged groups were required together for abscess induction, and although no specific type of negatively charged group was necessary, the presence of a non-acetylated free amino group was required.

A potential major flaw in this work is that the authors have not considered that they have probably extracted and analysed CP from a heterogeneous cell population. MAbs raised against PSA and PSB only partially cross-reacted with CP extracted from other *B. fragilis* strains (Pantosti *et al* 1992, Pantosti *et al* 1993), which suggests antigenic variation. Moreover, in these studies it was quoted that PSA and PSB exhibit variable expression on the cell surface of *B. fragilis* NCTC 9343, and that this explains the antigenic differences between LC, SC and EDL populations as observed by Patrick & colleagues. However, it is equally possible that the CP of some cells does not consist of PSA and PSB, or that other polysaccharides besides these are present.

1.4.1b Bacteroides Lipopolysaccharide

Bacteroides fragilis Lipopolysaccharide: Rough or Smooth?

Controversy surrounding bacteroides surface polysaccharides does not stop with CP; there has been an ongoing debate for years as to whether the LPS of *B. fragilis* is rough or smooth. The confusion in this case has largely arisen due to different extraction techniques employed to obtain the LPS. *B. fragilis* LPS was originally reported to be rough (Kasper *et al* 1983, Weintraub *et al* 1985), but it was obtained using the phenol / chloroform / petroleum ether (PCP) extraction method of Galanos *et al* (1969) which is selective for rough LPS. Moreover, Kasper *et al* (1983) assumed that the surface polysaccharides of *B. fragilis* were composed simply of CP and LPS, which could be easily separated as, for example, those of *E. coli* can. However, Cousland & Poxton (1984) demonstrated by crossed immunoelectrophoresis that the surface polysaccharides of *B. fragilis* were structurally and antigenically very complex. Subsequently, ladder patterns characteristic of smooth LPS were observed by polyacrylamide gel electrophoresis (PAGE) and immunoblotting of some *B. fragilis* strains (Poxton & Brown 1986). This was achieved by using aqueous phenol, which selectively extracts smooth LPS (Westphal & Luderitz 1954). In addition, a common antigen band was observed in all strains, migrating behind the lipid A and core regions which had not been observed in PCP extracts. Further reports of smooth LPS in *B. fragilis* have since been published; ladder patterns have been observed in LPS from the NC cell population of *B. fragilis* NCTC 9343 using MAbs (Lutton *et al* 1991), and in some *B. fragilis* strains by silver staining (Maskell 1994).

Bacteroides fragilis Lipid A Structure: Relation to Biological Activity

The structure of *B. fragilis* lipid A has been elucidated. It is markedly different from *E. coli* lipid A (Figure 5), and has a lower biological activity than that of *E. coli*. The differences in biological activity are thought to be a direct result of differences in

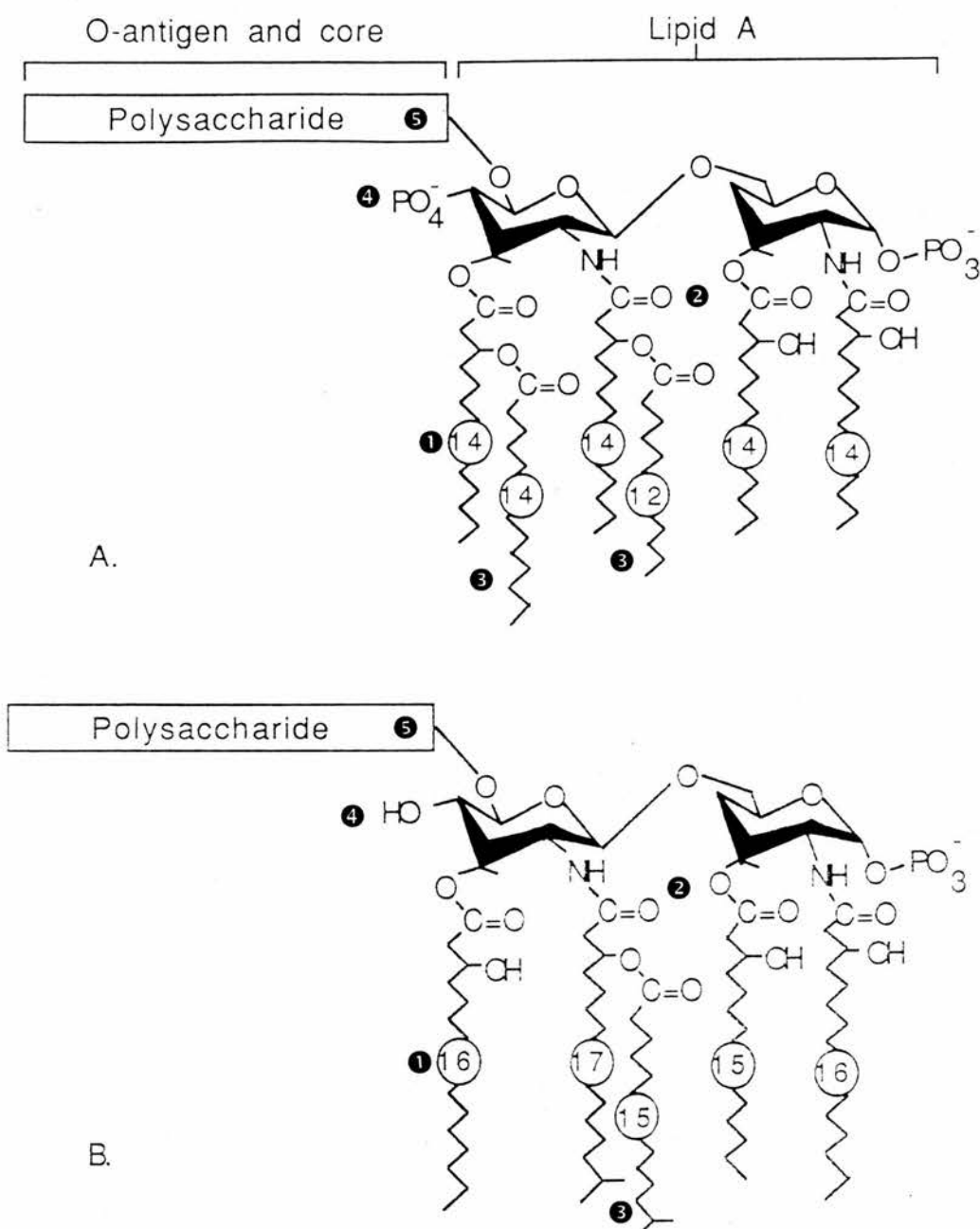


Figure 5. Differences in lipid A structure of *E. coli* (A) and *B. fragilis* (B): **1** the fatty acids of *B. fragilis* lipid A have chain lengths of 15-17 carbon atoms, whereas those of *E. coli* have 12-14, **2** there are 4-5 fatty acids per diglucosamine residue in *B. fragilis* lipid A, c.f. 6 in *E. coli*, **3** there is one β-hydroxy fatty acid substituted with a non-hydroxylated fatty acid in *B. fragilis* c.f. two in *E. coli*, **4** *B. fragilis* lipid A has one phosphate residue c.f. two in *E. coli* and **5** (not illustrated) the KDO of *B. fragilis* is phosphorylated whereas that of *E. coli* is not. From Magnuson *et al* (1989).

structure. The degree of difference is yet another matter of controversy; Lindberg *et al* (1990) found the biological activity of PCP-extracted LPS to be 100- to 1000-fold less than that of *E. coli* in various *in vivo* and *in vitro* assays. In contrast, a recent study in our laboratory found *B. fragilis* LPS to be more biologically active than previously thought, and degree of biological activity was highly dependent on extraction technique (Delahooke *et al* 1995a). LPS was extracted from bacteria using three different techniques: Triton-Mg²⁺ (Uchida *et al* 1987: mild, non-selective extraction), PCP and aqueous phenol. The Triton-Mg²⁺ and PCP methods both gave low biological activities in various assays, comparable to previously published reports. However the aqueous phenol extracts showed much higher activity, with *B. fragilis* being up to 30-fold more active than *E. coli* in the limulus amoebocyte lysate chromogenic (LAL) assay, and 4-fold less active than *E. coli* in a TNF induction assay.

Lipopolysaccharide of *Bacteroides* species other than *Bacteroides fragilis*

There has not been a vast amount of work carried out on the LPS of *Bacteroides* spp. other than *B. fragilis*. *B. fragilis* LPS has been reported to be more biologically active in a range of assays than other *Bacteroides* spp., which is in keeping with its greater pathogenicity (Delahooke *et al* 1995a). Resolution of bacteroides LPS by PAGE often proves difficult due to problems in separating the surface components, meaning that material staining on gels may not necessarily be LPS but could be residual protein or CP. Electrophoretic analysis of bacteroides LPS has shown *B. vulgatus*, and some strains of *B. stercoris* and *B. eggerthii* to produce ladder patterns characteristic of smooth LPS. The LPS profiles of *B. thetaotaomicron*, *B. caccae* and *B. ovatus* have been shown to be very similar, and although some minor inter-strain variations were seen, most species generally showed a species-specific profile (Maskell 1991, Maskell 1994). There have not been any reports published of variation in bacteroides LPS expression in different growth environments.

1.4.2 RESISTANCE TO COMPLEMENT

Previous studies have shown that *Bacteroides* strains isolated from infections are generally more resistant to complement than those isolated from faeces (Casciato *et al* 1975, Casciato *et al* 1979), suggesting that complement resistance is an important virulence factor for *Bacteroides* spp. In addition, clinical isolates of *B. fragilis* were found to be more resistant to complement than clinical isolates of other *Bacteroides* spp. (Casciato *et al* 1979, Rotimi & Eke 1984), which represents another factor contributing to the greater pathogenicity of *B. fragilis*. Another study found complement resistant strains of *B. fragilis* to survive better than complement sensitive strains in a subcutaneous model of infection (Namavar *et al* 1991). However, the mechanism of complement resistance in *Bacteroides* spp. is unknown. Reid & Patrick (1984) compared the ability of capsulate and non-capsulate *B. fragilis* strains to resist complement. It was found that possession of a capsule did not confer complement resistance, and in one strain tested, the non-capsulate cells were significantly more resistant than capsulate cells. This could be because the non-capsulate cells excrete CP as cell-free slime (Lutton *et al* 1991) which could theoretically mop up complement components at a distance from the bacterial cells, thereby preventing their deposition on the cell surface. It has been shown that complement resistance in *B. fragilis* and *B. vulgatus* is not due to lack of C3 deposition on the cell surface (Vel *et al* 1986), and in *B. fragilis* and *B. thetaiotaomicron* it is not due to lack of activation of the alternative complement pathway (Bjornson *et al* 1987). However, the roles of LPS, OMPs, other bacterial factors and host factors such as classical complement pathway activation in complement resistance of *Bacteroides* spp. have not been investigated. Moreover, complement resistance of strains grown in different environments (which can significantly alter cell surface antigen expression) have not been investigated.

1.4.3 NUTRITION *IN VIVO*

Any bacterium which escapes from the gut will find that nutrients are much less abundant elsewhere in the body. In order to survive, pathogenic *Bacteroides* spp. must be able to obtain nutrients at the infected site. This is achieved in two ways described below.

1.4.3a Release of Extracellular Enzymes

Bacteroides spp. have been found to release extracellular enzymes including hyaluronidase, DNase, lipase, proteases and neuraminidase, all of which are capable of breaking down host tissues and cells (Rudek & Haque 1976). The fact that *Bacteroides* spp. in infected sites do not cause extensive, fast destruction of host tissue suggests that the activity of these enzymes is localised (Patrick 1993). The breakdown products are probably taken up into the cell and used as a nutrient supply.

1.4.3b Outer Membrane Protein Expression and Iron Uptake

In order to survive in a new environment, a bacterium must be able to change OMP expression appropriately to obtain nutrients. It therefore might be expected that the OMP profile of any given strain grown *in vitro* would be different to the profile *in vivo*. However, a study which compared OMPs of *B. fragilis* grown either in broth culture or in chambers in mouse peritoneal cavities did not reveal any major differences in OMP expression (Patrick & Lutton 1990). It is possible in this case that some subtle differences were not picked up as Coomassie blue was used to stain the proteins, which is not particularly sensitive.

The OMP profiles of *Bacteroides* spp. have been shown to be much more complex than those of *E. coli* (Diedrich & Martin 1981, Kotarski & Salyers 1984). Several bacteroides OMPs have been identified. The first to be identified was a porin from *B. distasonis* (Wexler *et al* 1992) and the second was a 44 kDa protein expressed by *B.*

fragilis under iron limitation (Otto *et al* 1990b). Recently, three porins have been identified in *B. fragilis*, all of which allow the diffusion of small ($M_r < 340\text{-}400$) molecules through the membrane (Kanazawa *et al* 1995). There is no evidence to date that *Bacteroides* spp. produce siderophores (Otto *et al* 1988), although they may be able to utilise those produced by other bacteria, and there is no evidence that they utilise iron bound to transferrin or lactoferrin. However it appears that the 44 kDa iron-regulated OMP is involved in uptake of haem (Otto *et al* 1990a), and research to date suggests that *B. fragilis* uses haem from haemoglobin as an iron source *in vivo*. Further supporting evidence for this is that Abs against the 44 kDa OMP have been found in patients with *B. fragilis* infections, demonstrating that the protein is expressed *in vivo* (Otto *et al* 1992), and *B. fragilis* has been recently reported to produce a haemolysin (Otto *et al* 1995).

1.4.4 FIMBRIAE

It has previously been reported that the ability of bacteria to agglutinate erythrocytes is sometimes associated with the presence of fimbriae (Hofstad 1984). Fimbriae were first recognised in *B. fragilis* when it was observed that some clinical unencapsulated strains were able to agglutinate erythrocytes (Pruzzo *et al* 1984). Moreover, the haemagglutinating strains were up to 20-fold more adhesive to epithelial cells, and also up to 7-fold more sensitive to phagocytosis than non-haemagglutinating strains (presumably due to increased adherence to phagocytic cells). All of these strains were shown by electron microscopy to possess fimbrial-like structures. A later study by Brook *et al* (1992) which looked at the percentage of *B. fragilis* strains with fimbriae isolated from different sites showed 6% from blood, 75% from abscesses and 69% from normal flora to possess fimbriae. These studies suggest that fimbriae, possibly in addition to capsule, are involved in adherence of *B. fragilis* to host cells, and are therefore important in attachment and colonisation of potentially pathogenic species. Furthermore, localised agglutination of erythrocytes may erect a barrier

between the bacterial cell and the host immune system, as does coagulase produced by *S. aureus*. In abscesses and the gut, the majority of strains expressed fimbriae, which would presumably localise them to the area and facilitate attachment to host cells, whereas in blood only 6% of strains expressed fimbriae, so attachment would be less efficient and systemic spread would be facilitated. Fimbriae have been looked for in all *Bacteroides* spp. (Brook 1992) but have only been detected in *B. fragilis*, which may partly explain why it is more closely associated with gut mucosa than other spp., and may represent another facet of its greater pathogenicity.

Attempts have been made to characterise fimbriae from *B. fragilis*. Van Doorn *et al* (1987) reported that fimbriae from *B. fragilis* were approximately 4 nm in diameter and were composed of subunits with molecular weights of 40 to 42 kDa. The expression of fimbriae appeared to be environmentally regulated, being repressed under iron limitation and at a growth temperature of 20°C. Subsequently Lutton *et al* (1989) visualised fimbriae from different *B. fragilis* strains grown *in vitro* by electron microscopy and immunoblotting. Electron microscopy showed fimbrial structures which are thought to be dehydrated capsular material on LC and SC, but not EDL cell populations. Immunoblotting with serum against purified fimbrial antigens detected fimbriae predominantly in SC cell populations, but in some strains fimbriae were also detected in LC and EDL populations. In addition, four *B. fragilis* strains were grown in chambers in mouse peritoneal cavities, and fimbrial antigen was detected in all strains by immunoblotting. Expression of fimbriae in this *in vivo* model is in agreement with results from the human study which showed a high percentage of strains from abscesses to express fimbriae (Brook *et al* 1992).

Studies on fimbriae of *Bacteroides* spp. are far from complete. Progress in this area has proved difficult due to problems in visualising fimbriae on cells, and a general lack of knowledge of growth conditions which favour the expression of fimbriae

(Patrick 1993). Moreover, although it is thought that *B. fragilis* fimbriae enhance attachment to host cells, the mechanism by which they do so is unknown, and whether they mediate attachment via specific host cell receptors as some other bacterial fimbriae do remains to be seen. Lastly, as was the case with capsule, it may transpire that species of *Bacteroides* other than *B. fragilis* also produce fimbriae.

1.4.5 ENTEROTOXIN

Enterotoxigenic *B. fragilis* strains were initially isolated from the faeces of lambs, calves, pigs and foals with diarrhoea. In 1987, Myers *et al* isolated enterotoxigenic strains from 8 of 44 humans with diarrhoea. Symptoms lasted from 1-4 weeks, and included watery diarrhoea, intestinal cramps, and infants additionally had pyrexia, vomiting and bloody diarrhoea. Subsequent studies revealed that enterotoxigenic *B. fragilis* appear to be carried in approximately 5-10% of the general population (Shoop *et al* 1990).

An enterotoxin from *B. fragilis* has been purified (Van Tassell *et al* 1992), which was found to produce both enterotoxic and cytotoxic effects. It has recently been shown to be a metalloprotease (Moncrief *et al* 1995). Clinical and faecal isolates of *B. vulgatus*, *B. thetaiotaomicron* and *B. distasonis* have also been examined for enterotoxin, but none were found to produce it (Van Tassell *et al* 1992).

Diarrhoea kills around 10 million people per year worldwide, and in many cases the cause is never discovered. It is possible that enterotoxigenic *B. fragilis* may be responsible for at least some of these deaths. Moreover, it is possible that some of the localised tissue damage seen in *B. fragilis* infections is due to enterotoxin. It would be interesting to find out how many *B. fragilis* strains isolated from abscesses and other infections were enterotoxigenic.

1.4.6 SUMMARY

Bacteroides spp., particularly *B. fragilis*, possess a lot of potential virulence determinants, but despite extensive research many unanswered questions remain. Confusion has mainly arisen due to the complexity of the surface antigens. Different virulence determinants are probably involved in pathogenesis at different stages of the infectious process, and it is likely that strains which are best able to co-ordinate virulence factor expression are the ones which will survive outwith the gut and cause infection.

1.5 POTENTIAL ROLE OF BACTEROIDES SPECIES IN SYSTEMIC INFLAMMATORY RESPONSE SYNDROME AND INFLAMMATORY BOWEL DISEASE

Bacteroides spp., especially *B. fragilis*, have been shown to be important pathogens which are frequently isolated from anaerobic infections. Despite this, there are some disease processes in which bacteroides could play a major role, but to date have been largely overlooked or dismissed as insignificant. Two examples of these are systemic inflammatory response syndrome (SIRS) and inflammatory bowel disease (IBD). A summary of current knowledge of SIRS and IBD is given below, and the possible role of bacteroides in these diseases is discussed.

1.5.1 SYSTEMIC INFLAMMATORY RESPONSE SYNDROME

1.5.1a Definition of Systemic Inflammatory Response Syndrome

SIRS is the latest in a long list of terms which have been used to describe a severe generalised illness which is the leading cause of death in intensive care patients (Bone 1993, Darville *et al* 1993). Other terms which have been used include sepsis, severe sepsis, septic shock and sepsis syndrome.

SIRS is diagnosed by the presence of two or more of the following manifestations (Bone *et al* 1992):

- Temperature $> 38^{\circ}\text{C}$ or $< 36^{\circ}\text{C}$
- Heart rate > 90 beats per minute
- Respiratory rate > 20 breaths per minute or $\text{pCO}_2 < 32$ mm Hg
- Leukocyte count $> 12 \times 10^9$ cells per litre or $< 4 \times 10^9$ cells per litre, or the presence of $> 10\%$ immature neutrophils

1.5.1b Aetiology and Pathology

SIRS occurs as a result of bacterial products (usually endotoxin [LPS]) entering the circulation and binding to host cells via specific receptors (CD14, plus possibly other receptors, in the case of LPS: Rietschel & Brade 1992). This binding stimulates certain host cells (macrophages, neutrophils, T-cells and endothelium) to produce inflammatory mediators such as cytokines, oxygen free radicals and lipids (including clotting factors). Complement is also activated. The concentration of mediators produced is dependent on both the amount and the biological activity of the endotoxin; a low concentration of endotoxin will result in a low-level, controlled inflammatory response which will neutralise the endotoxin and kill any bacteria present. On the other hand, a high concentration of endotoxin will initiate production of an uncontrolled cascade of inflammatory mediators, resulting in a systemic inflammatory response (i.e. SIRS) which frequently leads on to shock, multiple organ failure (MOF) and death (Figure 6).

It is worth mentioning at this point that some researchers use the term "SIRS" to define the above illness when there is no obvious Gram-negative infection detectable, but use the term "sepsis" to define the same illness when Gram-negative infection is present (Mercier 1993, Bone 1995). Other authors use the two terms synonymously (Evans & Cohen 1993, Lynn & Cohen 1995), with the result that some of the literature on this subject is confusing to read (Gibb 1993).

1.5.1c Incidence of Systemic Inflammatory Response Syndrome

The number of deaths attributable to SIRS may be underestimated, as it is not a reportable disease (Bone 1993). Nevertheless, the mortality rates are extremely high; SIRS is reported to be the thirteenth leading cause of death in the USA (Darville *et al* 1993), with the number of deaths estimated at between 300,000 to 400,000 per year (Bone 1993, Lynn & Cohen 1995). There are large discrepancies in the estimates of

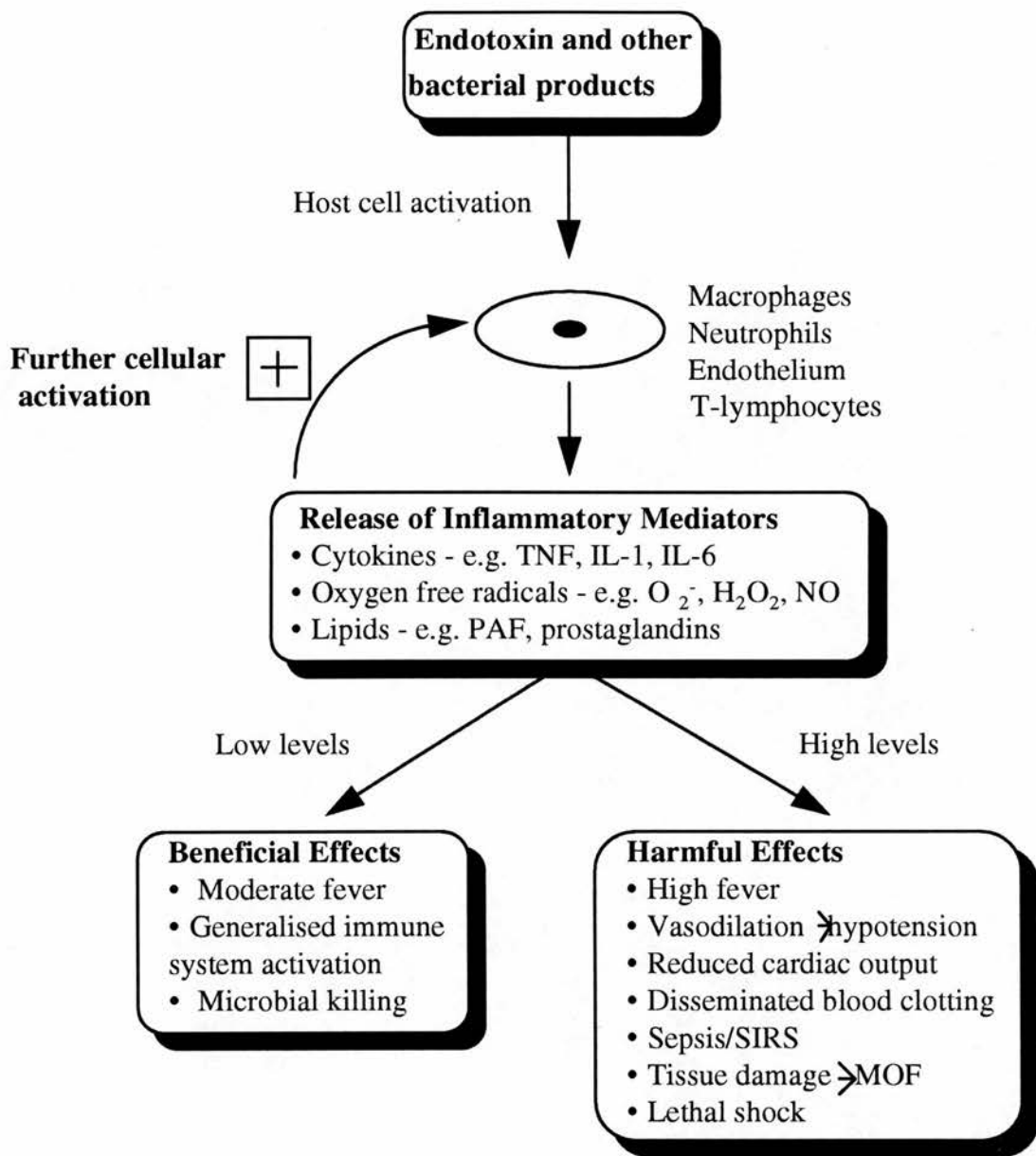


Figure 6. Sequence of events following host cell stimulation by endotoxin. High levels of proinflammatory mediators lead to the development of sepsis or SIRS. Abbreviations: IL = interleukin; PAF = platelet activating factor. Adapted from Rietschel & Brade (1992) and Lynn & Cohen (1995).

mortality rates due to SIRS, probably because of differences in definitions of the disease, because it is not a reportable disease and because the original diagnosis may be recorded as the cause of death rather than SIRS. Mortality rate estimates range from 20 to 60% (Bone 1993, Corriveau *et al* 1993, Evans & Cohen 1993), with mortality after the onset of shock greater than 40% (Darville *et al* 1993) and with shock plus MOF as high as 90% (Bone 1993).

1.5.1d Translocation

It would be a reasonable assumption to make that the endotoxin responsible for SIRS must be derived from an infection. However, previous studies have shown Gram-negative bacteraemia in only 12% (Willats *et al* 1994) to 37% (Gibb 1993) of SIRS patients, and in many SIRS patients an infectious focus is never found, even at post-mortem (Marshall *et al* 1988). It was first suggested over 30 years ago that in patients where there is no obvious Gram-negative infection, the source of endotoxin could be the intestinal flora (Fine *et al* 1959), and over the last several years this idea has gained popularity (Marshall *et al* 1988, Runcie & Ramsay 1990). The gut wall normally acts as a highly effective barrier, preventing the systemic spread of intestinal bacteria and their products. However, if the gut wall becomes ischaemic and starts to break down, its barrier function is reduced, theoretically allowing bacteria and / or their products to enter the circulation via the mesenteric lymph nodes and the portal vein - a process termed translocation (Wells *et al* 1988, Runcie & Ramsay 1990).

Studies measuring antibody levels to the LPS of various intestinal bacteria suggest that translocation may occur in healthy individuals. It has been found that healthy individuals have endogenous Abs against LPS core from a range of Enterobacteriaceae and closely related spp. (Barclay & Scott 1987, Barclay 1990). The levels of Ab vary between different individuals but they appear to be present in



everyone, suggesting that all individuals must have, or have had, enterobacterial LPS in their circulation at some stage. The most likely source of this LPS is the gut. However, to date, it has not been conclusively proved that bacteria and / or LPS translocate across the human gut wall. One recent study detected bacteraemia in two out of fifty patients following colonoscopy, but both had obstructing bowel cancer which could have significantly altered mucosal barrier function (Schoeffel *et al* 1994). Despite the lack of sound evidence in humans, translocation of bacteria (Mainous *et al* 1991, Wells *et al* 1992, Katouli *et al* 1994) and inert beads (Mora *et al* 1991) has been shown to occur in experimental animals. How relevant this is to the human situation remains to be seen.

Some of the circumstances under which gut barrier function has been shown to be reduced are as follows (Mainous *et al* 1991):

- Haemorrhagic shock
- Thermal injury
- Physical disruption of the gut mucosa
- Total parenteral nutrition
- Malignant disease of the gut
- Disruption of the ecological equilibrium of the intestinal flora resulting in bacterial overgrowth
- Impaired host immune defences

In studying this list, and assuming that the theory of translocation is correct, it becomes apparent why intensive care patients are so at risk from SIRS. Many intensive care patients have been involved in major accidents, suffered extensive burns and / or undergone major surgery. All of these would result in large fluid losses. When blood volume is severely depleted, the body responds by directing the

remaining blood to the "vital areas" - the heart and brain, and away from the "non-vital areas" - the gut and peripheries (Guyton 1992). The lack of oxygen to the gut causes the ischaemia and mucosal barrier breakdown. In addition, many intensive care patients receive total parenteral nutrition, meaning that the gut is not being used and so its blood supply is reduced, many are on antibiotic therapy which may change the balance of intestinal flora, and many are immunocompromised.

1.5.1e Treatment of Systemic Inflammatory Response Syndrome

Despite recent advances in technology and drug therapy which have resulted in great improvements in management of critically ill patients, mortality attributable to SIRS has changed little over the last 20 years (Lynn & Cohen 1995). A vast array of treatment strategies have been, and are currently under investigation. Many have given promising results in animal models, but unfortunately these have not been repeated when applied to humans. Approaches for treatment of SIRS fall into three broad categories: prevention of host cell activation, inhibition of host inflammatory mediators and limitation of organ damage. These are discussed in turn below.

Prevention of Host Cell Activation

Treatment strategies to prevent host cell activation have centred around the interaction of host cells with endotoxin. As this is the primary event in the pathogenesis of SIRS, these treatments are likely to be of most benefit when administered either early in the disease process, or when given prophylactically to high risk patients. Various methods have been suggested to identify high risk patients. Obviously, any critically ill patient who has suffered significant fluid loss and / or has a Gram-negative infection is high risk. Some patients diagnosed as having SIRS by the standard criteria will have much milder disease than others, so a severity-of-illness scoring system will help to identify those at greater risk. Previous studies have suggested that individuals who have low endogenous levels of anti-

enterobacterial LPS core Abs have a poorer survival rate from SIRS than those who have high levels (McCabe *et al* 1972, Windsor *et al* 1993, Barclay 1994). It has therefore been suggested that measurement of anti-LPS Abs may be a means of identifying high risk patients. How useful this is in practice is debatable, as by the time a patient arrives in intensive care, they are invariably very sick, and their Ab levels at this stage may bear no resemblance to those in health. It has also been suggested that people with poor mucosal immunity may be at high risk of developing SIRS, as they would be less able to prevent translocation than people with normal mucosal immunity (Ferguson *et al* 1994). This would, however, be very difficult to measure and assess in practice.

The treatment strategy to prevent host cell activation which has received the most attention is the administration of anti-LPS Abs. These should bind to and neutralise excess LPS, thereby preventing it from activating host cells. Various antibodies, both monoclonal and donor-derived, have been tested for their ability to treat SIRS. Many of these have been specific for the lipid A and core regions, as these are the most conserved regions of the molecule, and so Abs against these regions should give a high degree of cross-protection. The most infamous of these was an ill-fated human monoclonal IgM named HA-1A, which bound specifically to lipid A from a range of enterobacteria and closely related spp. (Teng *et al* 1985). It was reported to reduce mortality in mice with SIRS (Teng *et al* 1985, Young *et al* 1989), but these studies should have been interpreted with more caution, as they were performed with ascites or hybridoma fluids, and not with purified Abs. The promising data from these studies resulted in the rapid progression of HA-1A to clinical trials. Initial trial results reported that it reduced mortality in SIRS patients (Ziegler *et al* 1991, Wortel *et al* 1992). However, subsequent re-analysis of these results suggested that the initial conclusions drawn were "over-optimistic" (Wenzel 1992), and whilst HA-1A reduced mortality in some categories of patients, it actually increased mortality in others

(Gibb 1993, Baumgartner 1994). This resulted in the withdrawal of HA-1A from trials and scared many drug companies which were developing other anti-LPS Abs into discontinuing research. Poor scientific research into HA-1A, followed by poor interpretation of data has caused major setbacks in this field, which is unfortunate, as other anti-LPS Abs could well prove useful in the treatment of SIRS (Dunn *et al* 1986, Cross *et al* 1993, Di Padova *et al* 1993, Bhattacharjee *et al* 1994, Di Padova *et al* 1994).

Other treatment strategies to prevent host cell activation include the use of MAbs to block CD14 (and therefore prevent LPS binding), LPS neutralising proteins and lipid A analogues which bind to CD14 but do not activate the host cells (Lynn & Cohen 1995).

One cautionary note with any anti-LPS treatments is that although it is assumed that LPS plays the pivotal role in SIRS, this has not been proven to always be the case, and if microbial products other than LPS were inducing SIRS in some patients, then in these cases anti-LPS therapy would be totally inappropriate. In order to make an accurate diagnosis to administer appropriate treatment, a rapid, reliable means of detecting LPS in blood is required. This often proves very difficult; when LPS enters the circulation it quickly becomes bound to host molecules, and is rendered undetectable by the LAL assay (Majde 1992, Yentis 1995). Barclay *et al* (1989) demonstrated that in some SIRS patients a sudden decrease in anti-enterobacterial LPS core Abs was concurrent with an episode of endotoxaemia (confirmed by LAL assays). In other cases there was consumption of Ab but no detectable endotoxin. However, endotoxin could have been present in these cases but undetectable by LAL. Measurement of anti-LPS Abs could therefore be a useful indirect method of detecting endotoxaemia. Measurement of urinary nitrate (which will be elevated during endotoxaemia due to nitric oxide released by activated host cells) has also

been suggested as an indirect method of detecting endotoxaemia and / or bacteraemia (Ingeborg *et al* 1994).

Inhibition of Inflammatory Mediators

Although SIRS can develop by many different mechanisms, cascades of inflammatory mediators are always produced as part of its pathophysiology. Therefore treatments aimed at neutralisation of proinflammatory mediators should be beneficial to all SIRS patients. Many of these have been aimed at TNF, as this is the first proinflammatory cytokine produced and is thought to play a pivotal role in induction of the cytokine cascade (Manthey & Vogel 1992). Some strategies include MAbs which bind to TNF or other proinflammatory mediators thereby neutralising their effects, the administration of soluble TNF receptors, which would mop up excess TNF and administration of antagonist molecules (e.g. interleukin-1-receptor antagonist) which bind to receptors and therefore prevent binding of inflammatory mediators (Lynn & Cohen 1995). The cautionary note in this case is that a low level of mediators is essential for the host to be able to deal with an infection and any LPS present. Therefore a balance must be maintained between effective inhibition of excessive host responses and the abolition of essential defences, which makes dosage decisions very difficult.

Limitation of Organ Damage

Much of the tissue injury that complicates SIRS results from the migration of activated neutrophils into tissues, which then release destructive enzymes and other destructive molecules. Strategies to limit this include inhibition of neutrophil migration, prevention of neutrophil activation, administration of scavenger molecules to mop up oxygen free radicals and protease inhibitors (Lynn & Cohen 1995).

In summary, treatment strategies for SIRS to date which have been successful in

animal models have generally given disappointing results when applied to humans. The main reason for this is probably that SIRS can be initiated in a multitude of ways, and a single strategy to treat such a complex disorder in a range of patients with complicated, totally different histories is unlikely to be successful. Reliance on animal models of SIRS, which are usually much less complex than the human situation, has led to incorrect conclusions being drawn about treatments. It is possible that SIRS will only be effectively treated by employing several different therapies at different stages of the disease process, which are tailor-made to suit the individual patient's circumstances.

1.5.1f Are Bacteroides Involved in the Pathogenesis of Systemic Inflammatory Response Syndrome?

It has previously been reported that anaerobes translocate very poorly in animal models (Wells *et al* 1988, Berg 1995), and that their presence in the gut actually reduces translocation of facultative bacteria (Wells *et al* 1987). However, it is possible that any translocated anaerobes had died by the time they were cultured from blood and were therefore not detected in these studies, and in addition translocation of LPS from anaerobic bacteria as opposed to whole, viable bacteria has not been looked at.

As previously discussed, in SIRS patients with no obvious Gram-negative infection the most likely source of endotoxin is the gut. It has previously been assumed that the endotoxin from the gut responsible for SIRS is that of *E. coli*, with anaerobic gut organisms not thought to be significant (Runcie & Ramsay 1990). However, although *E. coli* LPS is up to 1000-fold more biologically active than that of *Bacteroides* spp., there are up to 1000-fold more strict anaerobes in the gut than facultative anaerobes. Given the differences in numbers between *E. coli* and bacteroides in the gut, there is potentially as much if not more biological activity

from bacteroides LPS as there is from that of *E. coli*.

1.5.2 INFLAMMATORY BOWEL DISEASE

IBD is the term used to describe collectively two distinct diseases - Crohn's disease (CD) and ulcerative colitis (UC). These diseases are idiopathic in nature, and many aspects of their pathogenesis are unclear. Both are progressive and go through acute exacerbation and remission stages.

1.5.2a Crohn's Disease

Description

CD is a non-specific inflammatory process affecting all layers of the intestinal wall (i.e. the mucosa, submucosa, muscle and connective tissue layers). It most commonly affects the ileum, but may occur in multiple segments and can affect any part of the gastro-intestinal tract. There is inflammation and thickening of the submucosa, and ulceration and hypertrophy of the muscle wall, which cause narrowing of the lumen of the intestine. As the ulceration heals, scar tissue formation prevents absorption of nutrients and also causes strictures.

Clinical Features and Complications

A person with CD has a history of general ill health, weight loss and anaemia. Intermittent, often severe colicky pain associated with diarrhoea may occur three or four times daily, and the stools often contain blood, mucus, pus and undigested food. Malabsorption of nutrients causes a deterioration in general health. Oedema and congestion can result in adhesions between loops of bowel or other abdominal organs, and lead to the formation of fistulae or abscesses. Intestinal obstruction may occur due to strictures (Chilman & Thomas 1981).

Treatment

There is no cure for CD, so treatment is aimed at alleviation of symptoms. Steroids are given during acute exacerbations to reduce inflammation. Antibiotics are given if there are any signs of infection and abscess formation. Antispasmodic drugs help to alleviate pain associated with diarrhoea. Severe exacerbations may require intravenous blood and fluid replacement. Complications such as intestinal obstruction, abscess or fistula formation will require surgery, and grossly affected segments of disease can be excised (Podolsky 1991b).

1.5.2b Ulcerative Colitis

Description

UC is an inflammatory disease involving the mucosa and submucosa of the large intestine. Initially the mucosa of the sigmoid colon becomes swollen and congested, and the disease may spread to other parts of the large intestine. The mucosa bleeds easily and patchy ulceration occurs where the mucous membrane has been eroded.

Clinical Features and Complications

The initial symptoms of UC are often vague, and present as abdominal discomfort, mild diarrhoea and blood in the stools. The symptoms then become more defined, with loose stools and frequent, urgent discharge of blood and mucus, which can result in severe blood and fluid loss. In acute exacerbations, the person suffers from weight loss, anaemia, pain, fever and general debility. Over time, the continuous ulceration and healing processes and formation of scar tissue cause loss of both elasticity and absorptive capability of the colon. Infection may occur, which will result in muscle tone loss, dilatation of the colon and risk of perforation. Malignant changes and tumour formation may occur when the disease is long-standing and severe (Chilman & Thomas 1981).

Treatment

Distressing diarrhoea can be controlled by drugs which will slow down the hyperactive colon. Blood and fluid losses during an acute exacerbation may require intravenous replacement. The non-steroidal anti-inflammatory drug sulphasalazine is given orally in periods of remission to prevent relapse, and in severe cases steroids may be prescribed. Surgery will be indicated if medical treatment fails to control the symptoms, and may involve subtotal or total colectomy (Podolsky 1991b).

1.5.2c Predisposing Factors to Inflammatory Bowel Disease

Epidemiological studies have implicated several factors which may predispose certain individuals or groups of individuals to IBD. There has been a steady rise in the incidence of CD in Western Europe and the USA over the last 50 years, which could be related, at least in part, to the post-war high fat, low fibre "Western diet". On the other hand, the incidence of UC has changed little over the same time period (Rose *et al* 1988, Sonnenberg 1990). Smoking is known to exacerbate CD, but it actually appears to alleviate the symptoms of UC (Boyko *et al* 1987, Calkins 1989). Genetic factors also appear to predispose people to IBD; there is increased incidence of IBD in certain races of people (e.g. Ashkenazi Jews: Podolsky 1991a), and also in first-degree relatives of affected people (Bennet *et al* 1991). In addition, a study carried out on twins has demonstrated that there is a much higher incidence of monozygotic twins both having CD than dizygotic twins (Tysk *et al* 1988). It has been suggested that IBD - UC in particular - is more likely to develop in people of a nervous, highly-strung disposition (Chilman & Thomas 1981). This is probably a somewhat unfair suggestion, as the nature of both of these diseases is likely to put a large amount of stress on anyone suffering from them.

1.5.2d Hypotheses for Aetiology and Pathology of Inflammatory Bowel Disease

In a healthy individual, when an Ag is encountered by the gut immune system, a dual

response occurs. Initially, an active local immune response occurs against the Ag, which includes Ab production (IgM then IgA), and a local inflammatory response (mediated by cytokines, complement, lipids and oxygen free radicals) which is down-regulated following clearance of the Ag. This down-regulation prevents immune-mediated damage to the gut wall. Subsequently, there is an antigen-specific suppression of the systemic immune system, including suppression of IgG, IgE and T cell immunity. This phenomenon, known as oral tolerance, is critical in preventing potentially damaging hypersensitivity and allergic reactions to frequently-encountered Ags (Ferguson *et al* 1994). The Ags which elicit oral tolerance are usually non-invasive and harmless. In people with IBD, there is a lack of down-regulation of the immune response, resulting in excessive proinflammatory mediator production (Mahida *et al* 1989, Ligumsky *et al* 1990). This abnormal immune response could happen in two fundamental ways (Podolsky 1991a):

- Antigenic material (e.g. dietary or bacterial Ags) could initiate an abnormal, prolonged response, due to a **defect in triggering** of the gut immune system.
- A normal response to Ags could be initiated, but this response could then be abnormally prolonged and / or severe due to a **defect in regulation** of the immune response.

At present, it is not known if one or both of these defects occur in IBD, or if some individuals have the first defect whilst others have the second. Whatever the cause is, the outcome is damage to the mucosa by immunopathological mechanisms causing inflammation, possible autoimmune effects and immune complex formation, and a vicious circle of chronic disease is established. Some possible causes of IBD are discussed below.

Genetic Defect

The fundamental cause of IBD is highly likely to be genetic. Mutated genes could encode an abnormal immune regulatory product (Podolsky 1991a), or a product that causes a structural alteration in the gastrointestinal tract (e.g. increased permeability: Gibson *et al* 1988, Olaison *et al* 1988), which would render it more susceptible to attack by infection, toxins or autoimmune action (Delpre *et al* 1989). Some factors which could trigger the expression of abnormal gene products, thereby causing exacerbations of disease, are as follows:

Inappropriate Response to Innocuous Antigens

As mentioned previously, oral tolerance normally develops to dietary and other commonly-encountered Ags. In IBD, there could be an abnormal, over-active response to these Ags, and tolerance would be reduced as a result. This would lead to the production of excessive Ab, including IgE, resulting in damage to tissue through immune complex formation and a hypersensitivity or allergic reaction in the gut. It has been shown that mast cells are degranulated at sites of active IBD in the gut, which supports this theory (Sommers 1966, Dvorak *et al* 1978, Fox *et al* 1993). Also, abnormally high levels of certain IgG subclasses have been found in both the serum and gut mucosa of people with IBD; CD is associated with high levels of IgG2, whereas UC is associated with high levels of IgG1 (MacDermott *et al* 1989).

Infection

It has been suspected that infectious agents may be responsible for CD and UC ever since they were first recognised. Dalziel (1913) noted that IBD was similar in pathology to a ruminant disease caused by infection with *Mycobacterium paratuberculosis*, and subsequently Crohn *et al* (1935) noted many similarities between CD and intestinal tuberculosis. Other infectious agents which have been implicated in IBD include certain pathogenic strains of *E. coli* (Burke & Axon 1978),

Yersinia enterocolitica (Ibbotson *et al* 1992), *Clostridium difficile* exotoxin (Trnka & Lamont 1981), *Chlamydia trachomatis* (Orda *et al* 1990) and *Saccharomyces cerevisiae* (Barnes *et al* 1990). However, efforts to find an infectious agent consistently associated with CD and / or UC have been unsuccessful, as the infectious agents above have also been isolated from some control volunteers who did not have IBD (Podolsky 1991a). It is possible that people with IBD have an abnormal immune response to certain infectious agents.

Normal Gut Flora

The mechanism by which the gut immune system distinguishes between normal flora and pathogens is unknown. It has been suggested that normal flora may be poorly immunogenic (Berg & Savage 1972), or oral tolerance is elicited in response to normal flora. However, both of these theories must be doubtful as normal flora elicit a large systemic immune response, and oral tolerance is usually only developed in response to non-invasive, harmless Ags. It is possible that a normally non-pathogenic microbe may become pathogenic in people with IBD, and elicit an abnormal immune response.

Gut flora secrete or contain various products which are capable of inducing a large inflammatory response - for example LPS, peptidoglycan and formyl peptides. It is therefore possible that in IBD an abnormal response is initiated to one (or more) of these products (Podolsky 1991a). Supporting evidence for this theory has come from a study investigating IgA against *S. minnesota* Rc LPS, both in serum and in the gut. It was found that both serum- and gut-associated IgA levels were significantly raised in people with CD compared to those with UC and healthy controls (Ferguson *et al* 1994). Although *S. minnesota* is not part of the normal gut flora, Abs against the rough core are highly likely to cross-react with *E. coli* LPS. In addition, Abs against epitopes common to both enterobacterial Ags and Ags on host cells have been found

in people with IBD (Roche *et al* 1985), which could cause autoimmune damage.

In summary, despite extensive investigations, an explanation for the cause of IBD remains elusive. This suggests that its pathogenesis is more complex than a single cause and effect relation, and is likely to be the result of genetic factors, certain triggers and modifying factors, which may vary between different individuals (Shanahan 1993). If the cause, and therefore a cure, for IBD is not found in the near future, it is possible that novel treatments such as neutralisation of proinflammatory mediators may prove useful; for example, an interleukin-1-receptor antagonist has been found to reduce inflammation in a rabbit model of colitis (Cominelli *et al* 1990).

1.5.2e Are Bacteroides Involved in the Pathogenesis of Inflammatory Bowel Disease?

To date, the vast majority of work carried out on bacterial involvement in IBD has centred around either pathogenic bacteria or gut-derived *E. coli*. However, anaerobic gut flora could be very significant in IBD. A study investigating the nature of the gut flora in people with CD showed that some have increased numbers of strictly anaerobic Gram-positive cocco-bacilli and Gram-negative bacilli compared to controls (Van der Merwe *et al* 1988). This was noted in some of these cases before the clinical onset of CD, suggesting that an altered gut flora is involved in the aetiology and pathology of CD, rather than a response to the disease. If gut bacteria or their products are responsible for IBD, then there is a vast pool of material from *Bacteroides* spp. capable of inducing an inflammatory response. Some experimental evidence implicating bacteroides in IBD is that germ-free guinea pigs fed *B. vulgatus* were found to develop colonic ulceration with and without carragenan treatment (Onderdonk *et al* 1981), and immunisation of normal guinea pigs with *B. vulgatus* prior to carragenan treatment and ingestion of this organism resulted in a more rapid development of ulceration (Onderdonk *et al* 1983). OMPs and LPS were

subsequently implicated as causative agents for this phenomenon (Breeling *et al* 1988). *B. fragilis* did not produce this effect. Human studies are necessary to assess the significance of these models in IBD.

AIMS OF THIS THESIS

- To assess the influence of growth environment on cell surface Ag expression in *Bacteroides* species.
- Where possible, to relate any observed changes in surface Ag expression to pathogenicity.
- To investigate the complement and antibody response to bacteroides, both in health and in selected diseases where bacteroides may be significant.
- To examine the cell surface polysaccharide complex of *B. fragilis* chemically and immunologically, in order to address some of the unresolved questions which surround this subject.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 BLOOD AND SERUM SAMPLES

Whole venous blood samples were obtained from healthy volunteers (work colleagues), from SIRS patients in Edinburgh Royal Infirmary and from IBD patients attending an outpatient clinic at the Western General Hospital, Edinburgh.

Serum samples were obtained from a random selection of blood donors attending the Scottish National Blood Transfusion Service, Edinburgh (SNBTS), and from abdominal SIRS patients who were blood culture negative (Scottish Sepsis Intervention Group).

2.1.2 SAMPLE PROCESSING

Serum to be used as a source of complement was obtained from whole venous blood by two different methods:

Method A: Freshly drawn blood was allowed to clot at 37°C for 30 min, then centrifuged at 4000g for 10 min. Sera were removed, centrifuged as before and the supernates were pooled (Herbert 1989).

Method B: Freshly drawn blood was left to clot overnight at 4°C. It was then placed at room temperature for 30 min before removal of serum. Sera were centrifuged at 4000g for 10 min before the supernates were pooled (Hudson & Hay 1989).

In both methods, half of the pooled sera obtained were divided into 1 ml aliquots into 1ml cryotubes (Nunc) and stored at -70°C until immediately before use, whilst the remaining sera were heat-inactivated (56 °C / 30 min) prior to storage.

Sera to be used in antibody studies were obtained from whole venous blood by Method A described above, or were supplied already fractionated. These were stored at -20°C until use, either in 1 ml cryotubes (healthy volunteers, SIRS patients and IBD patients), or in 96-well sealed microtitre plates (Greiner; random blood donors).

2.1.3 BACTERIAL STRAINS

Bacterial strains used in this thesis are listed in Table 1a (*Bacteroides* strains) and in Table 1b (other bacterial strains).

2.1.4 CHEMICALS

Unless otherwise stated all chemicals were AnalR grade (BDH) and were prepared in distilled water (Milli-Q water purification system).

2.1.5 MEDIA

All media were prepared using pyrogen-free (PF) water (Milli-Q).

2.1.5a *Bacteroides* Strains: Complex Media

- **Proteose peptone-yeast extract medium (PPY).** This was prepared as previously described (Holbrook *et al*, 1977). The proteose peptone and yeast extract were from Oxoid.
- **Heat-inactivated sheep serum (HISS).** Filter-sterilised sheep serum was obtained from the Moredun Animal Diseases Research Institute, Edinburgh. Prior to using it for bacterial growth it was heat-inactivated (56°C / 30 min). In certain cases a

mixture of 50% HISS and 50% Van Tassell and Wilkins' minimal medium (described below) was used for bacterial growth.

- **Columbia blood agar (CBA: Oxoid).** Columbia agar base was supplemented with 5% horse blood.

2.1.5b *Bacteroides* Strains: Defined Medium

- **Van Tassell and Wilkins' minimal medium (VT)** was prepared as previously described (Van Tassell & Wilkins, 1978). For some experiments (indicated in relevant results sections) 300 μ M ethylenediaminetetra-acetic acid (EDTA), 450 μ M ethylenediamine-N,N'-diacetic acid (EDDA) or 0.5 mM ethyleneglycol-bis (β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) were added.

2.1.5c Other Bacterial Strains: Complex Media

- **Nutrient broth (NB: Gibco)** was prepared at the SNBTS Protein Fractionation Centre, Edinburgh.

2.1.6 MISCELLANEOUS

Sheep erythrocytes and anti-sheep erythrocyte IgG for determination of the haemolytic value of complement (CH50) were obtained from the Scottish Antibody Production Unit (SAPU), Law Hospital, Lanarkshire.

Polyclonal antiserum to *Bacteroides* strains was originally prepared in New Zealand rabbits, and was donated by Dr. I. R. Poxton (Dept. of Medical Microbiology, Edinburgh University) and by Dr. S. Patrick (Dept. of Microbiology and Immunology, Queen's University, Belfast). Murine MAbs (produced from hybridoma cell lines) against *B. fragilis* NCTC 9343 cell surface Ags were donated by Dr. S. Patrick, and are listed in Table 2.

Table 1a. *Bacteroides* Strains

Species	Strain No.	Source
<i>B. fragilis</i>	NCTC 9343 (T)	Appendix abscess
<i>B. fragilis</i>	MPRL 1504	Wound swab
<i>B. fragilis</i>	MPRL 1652	Faeces
<i>B. fragilis</i>	MPRL 1978	Blood
<i>B. fragilis</i>	NCTC 9344	Wound swab
<i>B. fragilis</i>	MPRL 1582	Unknown
<i>B. fragilis</i>	MPRL 1583	Unknown
<i>B. fragilis</i>	MPRL 1584	Rectal abscess
<i>B. fragilis</i>	MPRL 1585	Rectal ulcer
<i>B. fragilis</i>	MPRL 1586	Abscess
<i>B. fragilis</i>	MPRL 1588	Wound swab
<i>B. fragilis</i>	MPRL 1590	Vaginal swab
<i>B. fragilis</i>	MPRL 1981	Blood
<i>B. fragilis</i>	MPRL 1986	Blood
<i>B. vulgatus</i>	NCTC 11154 (T)	Unknown
<i>B. vulgatus</i>	MPRL 1651	Faeces
<i>B. vulgatus</i>	MPRL 1985	Blood
<i>B. thetaiotaomicron</i>	NCTC 10582 (T)	Faeces
<i>B. thetaiotaomicron</i>	MPRL 1959	Blood
<i>B. distasonis</i>	ATCC 8503 (T)	Unknown
<i>B. distasonis</i>	MPRL 1522	Faeces
<i>B. ovatus</i>	ATCC 8483 (T)	Unknown
<i>B. ovatus</i>	MPRL 2370	Blood
<i>B. uniformis</i>	ATCC 8492 (T)	Unknown
<i>B. uniformis</i>	MPRL 1542	Faeces
<i>B. uniformis</i>	MPRL 1987	Blood
<i>B. variabilis</i>	VPI 11368 (T)	Unknown
<i>B. variabilis</i>	MPRL 2244	Unknown
<i>B. eggerthii</i>	NCTC 11155 (T)	Faeces
<i>B. eggerthii</i>	MPRL 1523	Faeces
<i>B. eggerthii</i>	MPRL 1216	Appendectomy
<i>B. caccae</i>	ATCC 43815 (T)	Faeces
<i>B. caccae</i>	MPRL 1555	Wound
<i>B. merdae</i>	ATCC 43814 (T)	Faeces
<i>B. stercoris</i>	ATCC 43813 (T)	Faeces

NCTC = National Collection of Type Cultures, UK; ATCC = American Type Culture Collection; VPI = Virginia Polytechnic Institute, USA, MPRL = Departmental stock culture; (T) = Type Strain.

Table 1b. Other Bacterial Strains

Species	Strain No.	Source
<i>Escherichia coli</i> K12	MPRL 1303	Dr. Nils Carlin, National Bacteriology Laboratory, Sweden.
<i>Salmonella typhimurium</i> Rc 878	MPRL 0947	Prof. I.W. Sutherland, Institute of Cell & Molecular Biology, Edinburgh University.
<i>Klebsiella pneumoniae</i> 10B	MPRL 0954	Dr. I.R. Poxton, Dept. of Medical Microbiology, Edinburgh University.
<i>Pseudomonas aeruginosa</i> PAC 611	MPRL 1091	Prof. Pauline Meadows, UCL, London.

Abbreviations as in Table 1.

Table 2. Murine monoclonal antibodies against *B. fragilis* NCTC 9343 cell surface Ags.

MAb name	Class	Epitope
3C8	IgG2b	O-Ag
3D7	IgG1	LC / EDL
4C5	IgG1	LC / EDL / slime
1A4	IgG1	99% of <i>B. fragilis</i> isolates
6G3	IgG2b	? Common Ag
3F6	IgG2a	Undefined non-capsular epitope on SC cells
3C10	NC	Undefined
1E2	NC	Undefined
2B8	NC	Undefined
2E12	NC	Undefined
5B7	NC	Undefined
3H5	NC	Undefined

NC = not classified

2.2 METHODS

2.2.1 MAINTENANCE AND GROWTH OF BACTERIA

Bacterial strains were obtained initially from freeze-dried stocks, and thereafter were maintained at -70°C in 10% w / v skimmed milk (Oxoid).

All bacteria were grown at 37°C. Lyophilised bacteria were resuspended in approximately 0.5 ml of PPY (*Bacteroides* strains) or NB (other strains) and were streaked onto CBA. Following growth, bacterial suspensions were prepared by emulsifying several single colonies in 0.8 ml of skimmed milk in 1 ml cryotubes.

Bacteria were streaked onto CBA from the -70°C stocks and were sub-cultured onto fresh plates within seven days. A maximum of three sub-cultures were performed after which fresh plates were prepared from frozen stocks.

Bacteria were inoculated into 10 ml of liquid medium from plates, which could then if required be used as a starter culture to inoculate into larger volumes.

2.2.1a *Bacteroides* Strains

Bacteroides strains were incubated either in an anaerobic cabinet (Forma) in an atmosphere of H₂ 10%, CO₂ 10% and N₂ 80%, or in anaerobic jars in an atmosphere of H₂ 90% and CO₂ 10%. All liquid media used for growth of *Bacteroides* strains was pre-reduced for 24-48 h prior to inoculation.

2.2.1b Other Strains

All other strains were incubated aerobically. Liquid cultures were incubated in an orbital incubator (Gallenkamp) at 100-120 rpm.

2.2.2 PURITY CHECKS

Purity checks were carried out for all bacterial cultures at each new inoculation stage and at the end of all experimental procedures. Cultures were examined by Gram's staining, and by aerobic and anaerobic incubation on CBA.

2.2.3 MEASUREMENT OF BACTERIAL CONCENTRATION

2.2.3a Viable Counts

Serial ten-fold dilutions of *Bacteroides* strains were prepared in phosphate-buffered saline (PBS: Oxoid), and 100 µl volumes were spread onto CBA plates. Plates were incubated for 48 h and colonies were counted.

2.2.3b Measurement of Optical Density

Optical density measurements were made at 600 nm in a spectrophotometer (Pye Unicam SP6-550 UV / VIS). The suspending medium was used as a diluent and as a blank for optical density (OD) readings. Standard curves of \log_{10} bacterial numbers (determined by viable counts) against OD_{600} was drawn. An OD_{600} of 0.15 is equivalent to a concentration of approximately 10^8 colony forming units (cfu) of *Bacteroides* strains per ml.

2.2.4 PREPARATION OF LIPOPOLYSACCHARIDE USING PROTEINASE K DIGESTION OF WHOLE BACTERIA

LPS was prepared for visualisation by PAGE and silver staining by digestion of whole bacteria with Proteinase K (Protease Type XI, Sigma). Overnight cultures (10 ml) of *Bacteroides* strains were subjected to Proteinase K treatment using the method of Hitchcock & Brown (1983) as described by Hancock & Poxton (1988). This method is non-selective, and can be used to prepare both S- and R-LPS.

2.2.5 PREPARATION OF LIPOPOLYSACCHARIDE USING A MIXTURE OF AQUEOUS PHENOL / CHLOROFORM / PETROLEUM ETHER

Two litre batch cultures of *E. coli*, *S. typhimurium*, *K. pneumoniae* and *P. aeruginosa* were harvested by centrifugation (10000g / 20 min), washed twice in PBS and lyophilised. Lyophilised cells were then used for the preparation of LPS by the PCP method of Galanos *et al* (1969), as described by Hancock & Poxton (1988). The resulting LPS obtained was lyophilised, and dry weights of both the cells and LPS were noted. LPS was resuspended to 1 mg / ml in PF water and stored at -20°C until just prior to use. The PCP method is selective for R-LPS.

2.2.6 PREPARATION OF LIPOPOLYSACCHARIDE USING AQUEOUS PHENOL

The aqueous phenol method of Westphal & Luderitz (1954) as described by Hancock & Poxton (1988) was used to extract LPS from 1 L batch cultures of selected *Bacteroides* strains. In certain cases (indicated in the relevant results sections) the final rotary evaporation and ultracentrifugation steps were omitted. Cells were harvested, washed and lyophilised as described for the PCP extraction, and the final LPS pellet was lyophilised. Dry weights of the cells and LPS were noted. Lyophilised LPS was resuspended to either 1 mg / ml or 10 mg / ml in PF water and stored at -20°C until just prior to use. The aqueous phenol method is reported to be selective for S-LPS.

2.2.7 PREPARATION OF LIPOPOLYSACCHARIDE USING A RAPID AQUEOUS PHENOL EXTRACTION METHOD

Overnight cultures (10 ml) of *Bacteroides* strains were used to prepare LPS for examination by PAGE by the micromethod of Fomsgaard *et al* (1993). This method is based on the aqueous phenol method of Westphal & Luderitz (1954).

2.2.8 PREPARATION OF OUTER MEMBRANE PROTEINS

OMPs were prepared from selected *Bacteroides* strains by a method based on that of Filip *et al* (1973). Batch cultures of bacteria (400 ml) grown to stationary phase in either PPY, VT or 50% VT / 50% HISS were harvested by centrifugation (10000g / 20 min), washed twice in PBS and resuspended to a final volume of 5 ml in PF water containing 0.1 mM phenylmethylsulphonyl fluoride. Cells were broken by sonication on ice (MSE soniprep) at an amplitude of 10 μ for a total of 5-7 min. Lysis was assessed by phase contrast microscopy. Unbroken cells were removed by centrifugation (4000g / 5 min), and the supernate was mixed with 30% w / v N-lauroyl sarcosinate (Sarkosyl) to give a final concentration of 0.7% w / v Sarkosyl. OMPs were sedimented by centrifugation (75000g / 1 h), washed once in 5 ml of PF water and re-centrifuged as before. The final pellet was resuspended in approximately 0.5 ml of PF water and stored at -20 °C.

2.2.9 FRACTIONATION OF THE SURFACE POLYSACCHARIDE COMPLEX OF *B. FRAGILIS* NCTC 9343

An attempt was made to separate and purify the different components of the surface polysaccharide complex (CP and LPS) of *B. fragilis* NCTC 9343 by a method based on that of Pantosti *et al* (1991). All reagents used in this procedure were made up with PF water. Cells were grown to stationary phase in 4 x 1 L batch cultures in PPY and then harvested by centrifugation (10000g / 15 min). The cells were washed twice in 0.15 M NaCl, the pellet was resuspended in 200 ml of PF water, and an equal volume of 75% aqueous phenol was added. The mixture was stirred at 68°C for 30 min, allowed to cool and then centrifuged (5000g / 20 min) to separate the aqueous and phenol phases. The phenol phase was then re-extracted as above. The two resulting aqueous phases were pooled, extracted with an equal volume of ether, left at 20°C overnight to remove the ether, dialysed extensively against PF water until the smell of phenol could no longer be detected, and lyophilised.

The lyophilised aqueous phase (2.45 g dry weight) was resuspended in 30 ml of 0.1 M sodium acetate buffer (pH 4.5) containing 10 mM CaCl_2 and 10 mM MgCl_2 . It was then treated with 0.5 mg DNase Type 1 (Sigma) and 2.5 mg RNase A (Sigma) at 37°C for 2 h. Toluene (100 μl) was added to the mixture to prevent bacterial growth. This treatment was repeated overnight. The pH was then adjusted to 7.0 using 5 M NaOH before 5 mg Pronase (BDH Biochemical Grade) was added twice, once for 2 h at 37°C and again overnight. Toluene was added as before, and removed following treatments by heating the mixture to 60°C. The material was brought to a concentration of 80% v / v ethanol and left at 4°C overnight. The alcohol-insoluble precipitate was recovered by centrifugation (10000g / 20 min) and dissolved in buffer (pH 9.8) containing 0.5% sodium deoxycholate, 50 mM glycine and 10 mM EDTA to a final volume of 9 ml. Three millilitres of this material were loaded onto a Sephacryl S-300 gel filtration column (1.5 x 60 cm: Pharmacia) which had been equilibrated in the same buffer as the samples. The fractions collected (1.8 ml; 250 ml in total) were examined by PAGE (10% separating gel, non-SDS; see Section 2.2.17). The fractions that contained high, intermediate and low *Mr* material were pooled separately, precipitated in 80% ethanol, dialysed against PF water and lyophilised. Prior to lyophilisation, the UV absorbance was measured at 280 nm to ensure that all traces of nucleic acids had been removed.

To achieve separation of the different components of the CP (PSA and PSB), 4.2 mg of the high *Mr* material was treated with 10ml of 5% acetic acid for 1 h at 100°C. The acetic acid was removed by rotary evaporation before the sample was lyophilised and resuspended in 5 ml of 50 mM Tris (2-amino-2-(hydroxymethyl) propane-1,3-diol)-HCl (pH 7.3) and loaded onto a DEAE-Sephacel (Pharmacia) ion-exchange column (1.5 x 15 cm) equilibrated in the same buffer. Buffer (100 ml) was washed through the column and 5 ml fractions were collected. The material bound to the column was eluted with a linear gradient of 0 to 0.5 M NaCl and collected in 5 ml

fractions (100 ml in total). Material obtained during the washing stage and at the low end (up to 0.1 M NaCl) of the gradient was pooled (PSA), and that obtained from the remainder of the gradient (> 0.1 M NaCl) was pooled separately (PSB). Both pools of material were lyophilised.

Following lyophilisation, samples were desalted by resuspending them in 5 ml PF water and running them in 200 μ l aliquots through disposable polystyrene chromatography columns (80 x 8 mm: Pierce & Warriner). The columns were packed with Sephadex G25 (Pharmacia) washed in PF water and calibrated with Blue Dextran 2000 (Pharmacia) prior to use. The desalted fractions were lyophilised.

All lyophilised samples obtained from the entire fractionation procedure (high, intermediate and low *Mr* material from the gel filtration column and PSA and PSB from the ion exchange and desalting columns) were resuspended to 1 mg / ml in PF water and stored at -20°C .

2.2.10 PERIODATE TREATMENT OF AQUEOUS PHENOL EXTRACTS OF LIPOPOLYSACCHARIDE

Aqueous phenol extracts of LPS from selected *B. fragilis* strains were treated with periodate to destroy carbohydrate. Aqueous phenol extracts (200 μ l) resuspended in PF water to 10 mg / ml were mixed with an equal volume of 0.1 M sodium periodate. The mixture was left in the dark overnight at 20°C . Ethylene glycol (50 μ l) was then added to remove the periodate before an equal volume of double strength PAGE sample buffer (see Section 2.2.14) was added. On addition of sample buffer the bromophenol blue turned yellow due to the acidic pH. Tris was added a crystal at a time until the solution turned blue. Samples were stored at -20°C .

2.2.11 PROTEINASE K TREATMENT OF AQUEOUS PHENOL EXTRACTS OF LIPOPOLYSACCHARIDE

Contaminating protein present in aqueous phenol extracts of LPS from selected *B. fragilis* strains was removed by Proteinase K treatment. Proteinase K (1.25 mg) was dissolved in 0.5 ml buffer (0.0625 M Tris-HCl with 2% w/v sodium dodecyl sulphate [SDS], pH 6.8). Aqueous phenol extracts (150 µl of a 10 mg / ml solution) were mixed with 60 µl of the Proteinase K solution before heating to 60°C for 1 h. Double strength PAGE sample buffer (90 µl) was added. Samples were stored at -20°C.

2.2.12 ESTIMATION OF PROTEIN CONCENTRATION

Protein concentration was estimated in selected LPS preparations, in surface polysaccharide fractions and in all OMP preparations using the Lowry assay (Lowry *et al* 1951).

2.2.13 ESTIMATION OF CARBOHYDRATE CONCENTRATION

Carbohydrate (i.e. neutral sugar) concentration was estimated in selected aqueous phenol extracts and in surface polysaccharide fractions using the method of Dubois *et al* (1956), as described by Hancock & Poxton (1988).

2.2.14 ESTIMATION OF ORGANIC PHOSPHORUS CONCENTRATION

Organic phosphorus was estimated in selected aqueous phenol extracts and in surface polysaccharide fractions using the method of Chen *et al* (1956), as described by Hancock & Poxton (1988).

2.2.15 ESTIMATION OF KDO CONCENTRATION

KDO was measured in selected aqueous phenol extracts and in surface polysaccharide fractions using a thiobarbiturate assay based on the method of Kharkanis *et al* (1978), as described by Hancock & Poxton (1988).

2.2.16 PREPARATION OF SAMPLES FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

PAGE sample buffer (pH 6.8) contained 0.0625M Tris with 2% w / v SDS, 10% v / v glycerol, 1% v / v 2-mercaptoethanol and 0.001% bromophenol blue. Double strength sample buffer (pH 6.8) contained the same constituents at double the concentrations.

All samples and *Mr* markers were boiled for 3 min after mixing with sample buffer just prior to adding to gels in order to re-dissolve precipitated SDS in the sample buffer.

2.2.16a Outer Membrane Protein Samples

OMP samples were mixed with an equal volume of double strength sample buffer. Samples were added to the gels at a concentration of 40 µg per track wherever possible. If the protein concentration of the sample was too low to achieve this, the highest concentration possible was added.

2.2.16b Lipopolysaccharide Samples

- Proteinase K samples were mixed with an equal volume of sample buffer and added to gels at 20 µl per track.
- Aqueous phenol, rapid phenol and PCP extracts were mixed with an equal volume of double strength sample buffer and added to gels at 20 µl or 40 µl per track (for 20-well gels) or at 1 ml per track (for 1-well gels).
- Periodate-treated LPS samples were added to gels at 40 µl or 80 µl per track.
- Proteinase K-treated aqueous phenol extracts of LPS were added to gels at 20 µl or 40 µl per track.

2.2.16c Surface Polysaccharide Fractions

Fractions obtained from the surface polysaccharide purification procedure (Section

2.2.9) from both the gel filtration column (high, intermediate and low *Mr* material) and from the ion-exchange column (PSA and PSB) were mixed with an equal volume of double strength sample buffer and added to gels at 40 µl or 80 µl per track (for 20-well gels) or at 1 ml per track (for 1-well gels).

2.2.16d Molecular Weight Standards

Mr standards (10 µl) were electrophoresed alongside antigen samples in selected cases. Standards (BDH Electrophoresis Grade) were reconstituted in single strength PAGE sample buffer following manufacturers' instructions and consisted of ovotransferrin, 78 kDa; albumin, 63 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 30 kDa; myoglobin, 17.2 kDa; cytochrome c, 12.3 kDa.

2.2.17 POLYACRYLAMIDE GEL ELECTROPHORESIS

PAGE was performed on acrylamide slab gels using the discontinuous buffer system of Laemmli (1970). The following solutions were used:

- **Separating buffer** (double strength, pH 8.8): 0.75 M Tris-HCl with 0.2% w / v SDS.
- **Stacking buffer** (double strength, pH 6.8): 0.25 M Tris-HCl with 0.2% w / v SDS. SDS was omitted from stacking and separating buffers used to electrophorese LPS and CP samples.
- **Acrylamide stock solution** (40% w / v): 100 g acrylamide (BDH Electrophoresis Grade) and 2.7 g methylene bis acrylamide (BDH Electrophoresis Grade) were made up to 250 ml in distilled water.

• **Electrode buffer** (pH 8.3): 0.025 M Tris (BDH Biochemical grade) and 0.192 M glycine (BDH Chromatographically Homogeneous) with 0.1% w / v SDS.

The separating gel was prepared as described in Table 3 and poured between glass plates (160 mm x 125 mm x 1.5 mm) using a needle and syringe. The gel was overlaid with water-saturated butan-2-ol and allowed to set. After removal of the butan-2-ol, the stacking gel (see Table 3) was poured onto the separating gel and either a 20-well or 1-well comb was inserted. The gel was then placed in an electrophoresis tank, the comb was removed and the electrode buffer was added.

Samples were loaded into the well(s) in the stacking gel. Samples were run through the stacking gel at 60 V and then through the separating gel at 150 V. Following electrophoresis, samples were visualised by staining the gel directly or by immunoblotting after transfer to nitrocellulose.

Table 3. Preparation of Polyacrylamide Gels

Reagent	Volume (ml) to give final acrylamide concentration of :			
	Separating gel			Stacking gel
	8%	10%	14%	4%
Distilled water	8.7	6.95	3.45	3.5
Separating buffer	17.5	17.5	17.5	-
Stacking buffer	-	-	-	5.0
40% acrylamide	7.0	8.75	12.25	1.0
TEMED*	0.05	0.05	0.05	0.02
Ammonium persulphate (15 mg / ml)	1.75	1.75	1.75	0.5

* N N N' N'-tetramethyl-1,2-diaminoethane.

2.2.18 SILVER STAINING OF POLYACRYLAMIDE GELS

Silver staining was used to visualise LPS, CP and some OMP samples by the method described by Hancock & Poxton (1988). This method is a modification of previous silver staining methods developed by Tsai & Frasch (1982), Oakley *et al* (1980) and Hitchcock & Brown (1983).

2.2.19 COOMASSIE BLUE STAINING OF POLYACRYLAMIDE GELS

OMP samples were visualised on gels using the alternative Coomassie Blue staining method described by Hancock & Poxton (1988).

2.2.20 IMMUNOBLOTTING

Separated Ags were transferred to nitrocellulose membranes (pore size 0.2 μm , Schleicher & Schuell) and then visualised by the method of Towbin *et al* (1979), as described by Hancock & Poxton (1988). Ags were probed with the following Abs for 3 h at room temperature:

- Human serum diluted 1 in 10
- Rabbit polyclonal hyperimmune serum diluted 1 in 100
- Murine MAbs - supernates from MAb-producing cell lines used undiluted

The immune complexes were detected with appropriate anti-IgG horseradish peroxidase (HRP) conjugates and HRP colour development reagent (BioRad). The following conjugates were used:

- Anti-human IgG (whole molecule) diluted 1 in 1000 (Sigma)
- Anti-rabbit IgG (whole molecule) diluted 1 in 1000 (Sigma)
- Anti-mouse IgG (whole molecule) diluted 1 in 500 (ICN)

2.2.21 DOT BLOTTING

Aqueous phenol extracts (10 mg / ml) and surface polysaccharide fractions (1 mg / ml) of *B. fragilis* NCTC 9343 were probed with polyclonal rabbit serum and murine MAbs by dot blotting on nitrocellulose, as described by Hancock & Poxton (1988). A total of 5 x 1 µl samples were placed onto the nitrocellulose for each antigen, allowing drying between each application. Immune complexes were visualised as for immunoblotting.

2.2.22 PERCOLL DISCONTINUOUS DENSITY CENTRIFUGATION

To assess the degree of encapsulation of *Bacteroides* strains grown in different environments Percoll (Pharmacia) discontinuous density centrifugation was used. Percoll was diluted as previously described (Patrick & Reid, 1983), and a step gradient was produced by layering 1 ml volumes of 80% (bottom), then 60%, 40% and 20% (top) Percoll into 70 x 20 mm glass test tubes. A sample of an early stationary phase culture of the test organism (1.25 ml) was applied to the top of the 20% layer and the gradient was centrifuged at 2600 g for 20 min.

2.2.23 INDIA INK STAINING

Cells were removed from the Percoll gradients following centrifugation and degree of encapsulation was examined microscopically using wet India Ink staining, as described by Cruickshank (1965).

2.2.24 COMPLEMENT SENSITIVITY ASSAY

Selected *Bacteroides* strains were grown to early stationary phase in 10 ml of PPY, VT and HISS or 50% VT / 50% HISS, and then tested for their ability to survive in human serum. Serum used for the assay was collected and stored as described previously (Section 2.1.2).

2.2.24a Determination of the Haemolytic Complement Value (CH50) of Serum

The CH50 value of all sera used as a source of complement was determined as previously described (Hudson & Hay 1980), except that PBS was used as a buffer instead of barbitone-buffered saline.

2.2.24b Serum Sensitivity Assay

Bacteria washed once in complement fixation test buffer (CFTB: Oxoid) were resuspended to a concentration of approximately 10^5 cfu / ml in either CFTB only (control), CFTB + 10% serum or CFTB + 40% serum and incubated aerobically (2 ml in 2.5 ml closed plastic tubes) at 37°C for 2 h with end-over-end rotation. Samples (100 µl) were taken at 0, 1 and 2 h in all cases and in some cases also at 20 and 40 min, diluted 1 in 50 in CFTB, and 100 µl of the resulting suspension were spread onto CBA in duplicate. After anaerobic incubation for 48 h, colonies were counted and the percentage survival was calculated compared to the start of the experiment. As a further control, all bacteria found to be sensitive to serum were resuspended to 10^5 cfu / ml in CFTB + 40% heat-inactivated human serum and treated as above. In a preliminary experiment, dilutions of bacteria were carried out in reduced (i.e. anaerobic) CFTB, and survival was compared with that in aerobic buffers. No differences were seen in bacterial survival.

2.2.25 CLASSICAL PATHWAY VERSUS ALTERNATIVE PATHWAY ACTIVATION OF COMPLEMENT

The roles of the classical and alternative pathways of complement activation were investigated in selected *Bacteroides* strains by two methods:

2.2.25a Absorption of Anti-bacteroides Antibodies from Serum

Serum was subjected to a series of absorptions with bacteroides whole cells to remove specific antibody (and therefore inhibit the classical pathway). Bacteria grown to

early stationary phase in PPY, VT or HISS were harvested and washed once in CFTB, and then resuspended in CFTB to approximately 10^8 cfu / ml. Bacterial suspension (1.0 ml) was added to a 1.5 ml Eppendorf tube and cells were harvested at 1365g in a microcentrifuge. After removal of supernatant, bacteria were resuspended in 1 ml of serum (thawed from -70°C then immediately placed on ice), incubated for 15 min on ice, and recentrifuged. The supernatant was then added to another pellet of cells and the process repeated. This step was repeated at least three times. Finally the serum was filtered ($0.2\ \mu\text{M}$, Millipore) to remove any bacteria still in suspension before using it in the complement sensitivity assay. As a control, a further 1 ml of serum was treated identically except no bacteria were added.

2.2.25b Use of EGTA to Inhibit the Classical Pathway

Serum was treated with EGTA to inactivate specifically the classical pathway by chelating Ca^{2+} ions but not Mg^{2+} ions as described by Fine *et al* (1979). A 200 mM stock solution of EGTA was made in normal (0.9%) saline and adjusted to pH 7.45 as described by Fine *et al* (1979). The EGTA stock was added to serum to a final concentration of 10 mM immediately prior to using it in the complement sensitivity assay.

2.2.26 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) REAGENTS

Polystyrene microwell strips (Immunomodule polysorp F8 Nunc, Intermed) fixed in frames to form 'plates' were used in ELISA experiments. Sterile, endotoxin-free glass tubes were used to make dilutions. All reagents and dilutions made for ELISA were done with PF water. The following diluents and buffers were used in the ELISA procedures:

- **Coating buffer** (pH 9.6): 0.05 M carbonate / bicarbonate with 0.02% w / v sodium azide.

- **Post-coat** (pH 7.2): PBS containing 5% v / v bovine serum albumin (BSA: ICN) and 0.02% w / v sodium azide.
- **Wash buffer** (pH 7.2): PBS containing 0.05% v / v Tween 20 (Sigma) and 0.05% w / v sodium azide.
- **Dilution buffer** (pH 7.2): PBS containing 0.05% v / v Tween 20, 0.5% v / v BSA, 4.0% w / v polyethylene glycol *Mr* 6000 (Sigma) and 0.02% w / v sodium azide.
- **Alkaline phosphatase substrate solvent** (pH 9.8): 0.05 M carbonate / bicarbonate buffer with 1 mM MgCl₂. Immediately prior to use, alkaline phosphatase substrate (p-nitrophenyl phosphate: Sigma) was added to a final concentration of 1 mg / ml.
- **Urease substrate solution** (pH 4.8). A stock solution of urease substrate (Sera-lab) was diluted 1 in 20 in water before adding to wells.

2.2.27 COATING OF LIPOPOLYSACCHARIDE ONTO ELISA STRIPS

2.2.27a *Bacteroides* Strains

Aqueous phenol LPS extracts resuspended to 1 mg / ml in water were complexed with an equal volume of a 1 mg / ml solution of polymyxin B sulphate (Sigma) after the method of Scott & Barclay (1987). The mixture was sonicated for 30 sec at 10 μ , stirred for 90 min at 20°C, then re-sonicated as above. Following dialysis for 18 h (Spectra por membrane MWCO 2000) against PF water at 20°C, the mixture was diluted 1 in 50 in coating buffer. The strips were coated at 100 μ l / well and incubated at 20°C overnight before being washed x 4 using an ELISA plate washer (Ultrawash 1, Dynatech). They were then post coated at 100 μ l / well and were incubated as above. Finally the strips were washed x 4 and rinsed x 1 in water.

2.2.27b Other Bacterial Strains

A mixture of equal weights of PCP-extracted *E. coli*, *S. typhimurium*, *K. pneumoniae* and *P. aeruginosa* rough LPS resuspended in water to 1 mg / ml was complexed with polymyxin B sulphate and coated onto ELISA strips as above.

2.2.28 COATING OF WHOLE BACTERIA ONTO ELISA STRIPS

Selected *B. fragilis* strains grown to early stationary phase in PPY, VT and 50% VT / 50% HISS were harvested by centrifugation (4000g / 10 min), washed once in PBS and resuspended in coating buffer to a concentration of approximately 2.5×10^7 bacteria per ml. Bacterial suspension (100 μ l) was added to ELISA strips, which were then centrifuged (630g / 4 min) to promote coating. Following overnight incubation at 20°C the procedure was as described for LPS-polymyxin coating.

In all cases coated ELISA strips were stored at -20°C for up to a maximum of three months.

2.2.29 DETECTION OF ANTI-BACTEROIDES AND ANTI-ENTEROBACTERIAL / PSEUDOMONAS LIPOPOLYSACCHARIDE ANTIBODIES IN HUMAN AND SHEEP SERUM BY ELISA

A 1 in 200 dilution of the serum samples to be tested was made in dilution buffer. The diluted serum (100 μ l) was added to each well on the appropriate LPS-coated ELISA strips and incubated for 90 min at 37°C before washing x 4. Either anti-human IgG (whole molecule: Sigma), anti-human IgG (γ -chain-specific: Bionetics) or anti-sheep IgG (whole molecule: Sigma), all conjugated to alkaline phosphatase, were diluted in ELISA dilution buffer 1 in 1000 and added at 100 μ l / well. The strips were incubated and washed as above. Alkaline phosphatase substrate was added at 100 μ l / well, the strips were incubated at 20°C for 30-40 min, and the absorbance at 405 nm was measured using an ELISA plate reader (Anthos 2001, Labtec). A

negative control was included where the serum was replaced by diluent, and the absorbance of the negative control was subtracted from each of the test wells.

2.2.30 INHIBITION ELISA USING LIPOPOLYSACCHARIDE TO ASSESS CROSS-REACTIVITY OF SERUM IgG TO DIFFERENT LIPOPOLYSACCHARIDES

This was carried out as above, except that after the serum was diluted 1 in 200, LPS was added to a final concentration of 1 mg / ml, and this was incubated at 37°C for 30 min prior to carrying out the ELISA. Control serum was treated identically except no LPS was added. Serum used in this case was pooled from 10 random blood donors.

2.2.31 INHIBITION ELISA USING WHOLE CELLS TO ASSESS CROSS-REACTIVITY OF SERUM IgG TO DIFFERENT LIPOPOLYSACCHARIDES

This was carried out as above, except that the serum was subjected to a series of absorptions with whole cells after being diluted 1 in 200 as described previously (Section 2.2.25a) prior to carrying out the ELISA. Serum used was pooled from 10 random blood donors.

2.2.32 DETERMINATION OF MEDIAN LEVELS OF ANTI-LIPOPOLYSACCHARIDE IgG WITHIN BLOOD DONOR POPULATION

Initially a sample of 50 random blood donors was screened by ELISA for IgG levels to *B. fragilis* NCTC 9343, *B. vulgatus* MPRL 1985, *B.thetaiotaomicron* NCTC 10582 and the enterobacterial / *Pseudomonas* LPS cocktail. To standardise OD readings, a control serum sample was included on each plate which had previously been shown (by Dr. R. Barclay) to have close to median levels of IgG against the enterobacterial / *Pseudomonas* R-LPS cocktail. When the OD of this sample had reached ~ 1, the plates were read. After this initial screen, serum containing close to median levels of IgG to all of the antigens was selected as a standard. A further 641

donors were then screened, and to standardise the OD readings, on each plate a sample of the standard serum was also tested. The OD of this serum was given an arbitrary value of 100% in each case, and the ODs of the 641 sera were converted to percentages using the standard serum. The median levels of IgG to all of the antigens within the 641 donors were calculated .

2.2.33 DETERMINATION OF ANTI-LIPOPOLYSACCHARIDE IgG LEVELS IN SYSTEMIC INFLAMMATORY RESPONSE SYNDROME PATIENTS AND INFLAMMATORY BOWEL DISEASE PATIENTS

ELISAs were performed on serum samples from 12 SIRS patients and 28 IBD patients. To standardise OD readings, another serum was selected as a standard from the 641 screened expressing close to median levels of IgG to all of the antigens and used as above. As a control for the SIRS patients' experiment only, serum taken from a healthy individual on six consecutive days was also tested.

2.2.34 COMPARISON OF BINDING OF IgG IN ELISA TO LIPOPOLYSACCHARIDE ALONE WITH LIPOPOLYSACCHARIDE COMPLEXED WITH POLYMYXIN B SULPHATE

LPS alone or polymyxin B sulphate alone were coated onto ELISA strips: LPS samples, or polymyxin B sulphate (both 1 mg / ml) were added to an equal volume of PF water, diluted 1 in 50 and coated onto strips at 100 µl / well as described above. Serum IgG levels from one SIRS patient were then measured in ELISA as described above, and compared to levels obtained with LPS complexed with polymyxin B sulphate.

To assess whether polymyxin B sulphate was preferentially binding certain parts of the LPS molecule, LPS was complexed with polymyxin B sulphate as described above, and the mixture was ultracentrifuged (100,000g / 3 h). The pellet and the

supernate were lyophilised, resuspended in PF water to 1 mg / ml and examined by PAGE and silver staining. As controls, uncomplexed LPS and polymyxin B sulphate alone (both resuspended in PF water to 1 mg / ml) were also examined by PAGE.

2.2.35 MEASUREMENT OF BINDING OF C3b TO WHOLE *BACTEROIDES FRAGILIS* CELLS IN ELISA

2.2.35a Treatment of Serum with Yeast Cell Walls

Prior to carrying out the ELISA, late complement proteins were depleted using yeast cell walls to activate the alternative complement pathway, so that remaining C3b would bind to bacterial cells but the cells would not lyse. Yeast cell walls (0.3 ml [0.5 mg / ml]; donated by Dr. J. Stewart) were washed once in Hanks' Balanced Salts Solution (HBSS: Gibco) , centrifuged (1365g / 3 min) and the pellet resuspended to a total volume of 1.5 ml. This was mixed with 1.5 ml human serum (collected by method A; Section 2.1.2) and incubated for 30 min at 37°C. Yeast cell walls and associated proteins were removed by centrifugation (630 g / 10 min).

2.2.35b C3b Binding ELISA

The yeast-treated serum was diluted further in HBSS to give a final concentration of 1% serum before being added at 100 µl / well to ELISA strips coated with whole bacteria. The strips were incubated at 37°C for 30 min and then washed x 4. An anti-human C3b murine MAb in ascites fluid (SAPU) was diluted 1 in 100, added to the strips at 100 µl / well and incubated at 37°C for 90 min. After washing x 4, a urease-conjugated polyvalent anti-mouse Ab (Sera-lab) was diluted 1 in 400 and added at 100 µl / well. After a further 90 min incubation, the strips were washed x 4, rinsed x 4 in water and developed with urease substrate solution (100 µl / well) for 30-40 min before OD was measured at 590 nm. A negative control was included where the serum was replaced by pure diluent, and the absorbance of the negative control was subtracted from each of the test wells.

CHAPTER 3

RESULTS

3.1 GROWTH OF *BACTEROIDES* SPECIES AND EXPRESSION OF CELL SURFACE ANTIGENS IN DIFFERENT ENVIRONMENTS

Alterations in growth environment can markedly change cell surface Ag expression, and these changes can be crucial in determining whether a potential pathogen will survive. The purpose of these experiments was to look at changes in bacteroides cell surface Ag expression in different growth environments. Three basic growth media were used: PPY (nutrient-rich), VT (minimal) and HISS ("physiological" medium).

3.1.1 GROWTH CURVES OF *BACTEROIDES* SPECIES IN DIFFERENT MEDIA

It was necessary to determine growth rates in the different media, so that bacteria could all be grown to approximately the same stage of the growth curve when cell surface Ags were examined.

Growth of the eleven *Bacteroides* type strains was measured over 24 h by OD and viable count (Figure 7). The growth curves of the type strains were taken to be representative of the growth of each respective species. In all cases, the most dense growth occurred in PPY. Some species grew better in VT than in HISS (e.g. *B. thetaiotaomicron*, *B. stercoris*), and the reverse was true for other species (e.g. *B. eggerthii*, *B. uniformis*). *B. vulgatus* did not grow in VT alone, and so for this species only, the VT was supplemented with 10% HISS. All other strains tested grew in VT, but in three cases (*B. caccae*, *B. eggerthii* and *B. distasonis*) the growth was poor. Although all of the strains tested grew in HISS, the only species to achieve dense growth in this medium was *B. fragilis*.

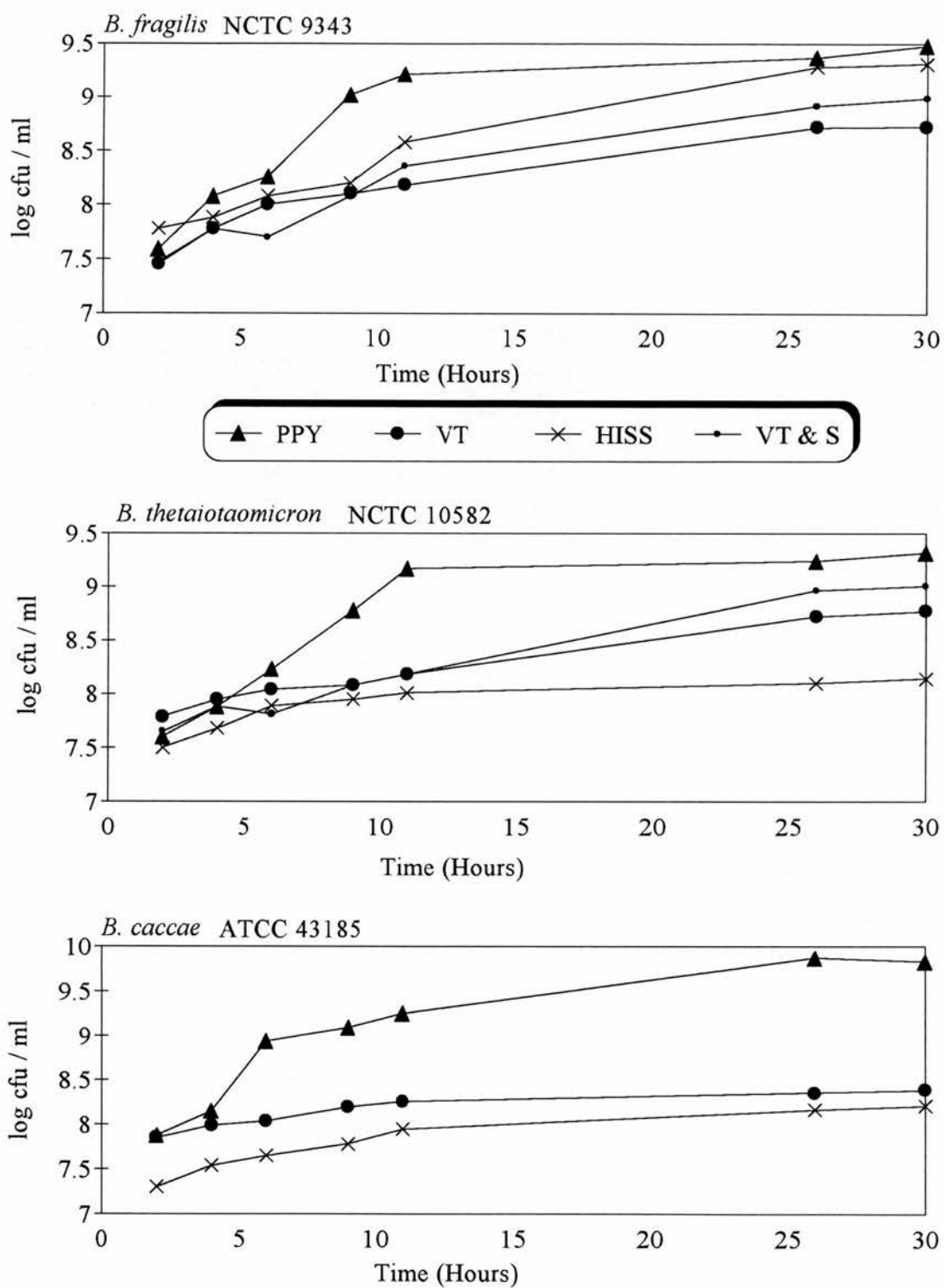


Figure 7. Growth curves of eleven *Bacteroides* type strains in different media.

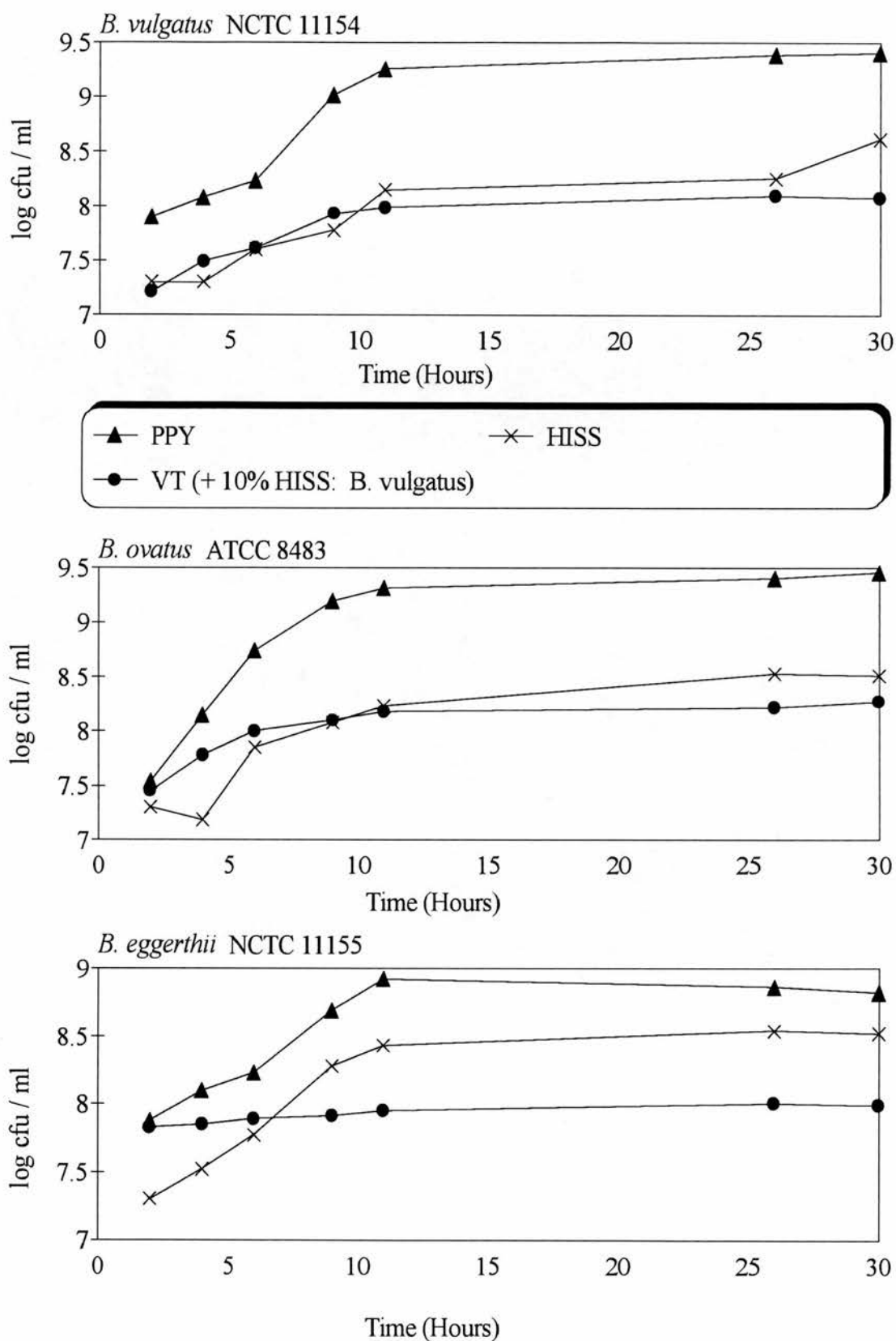


Figure 7 (continued).

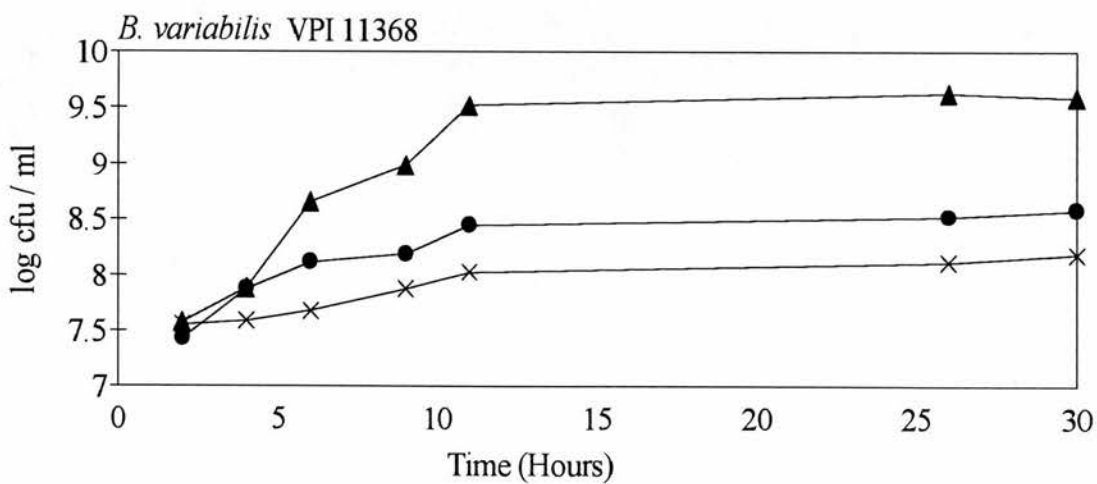
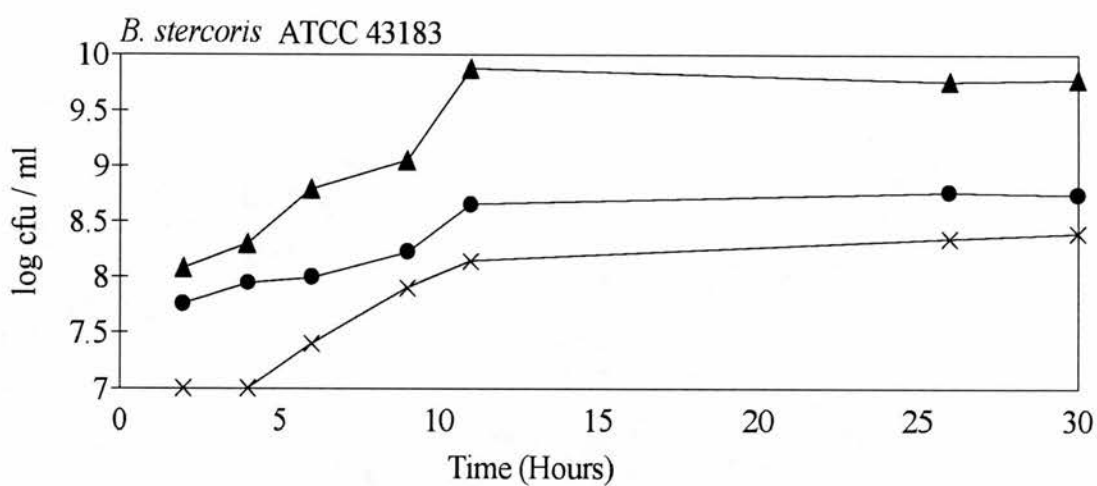
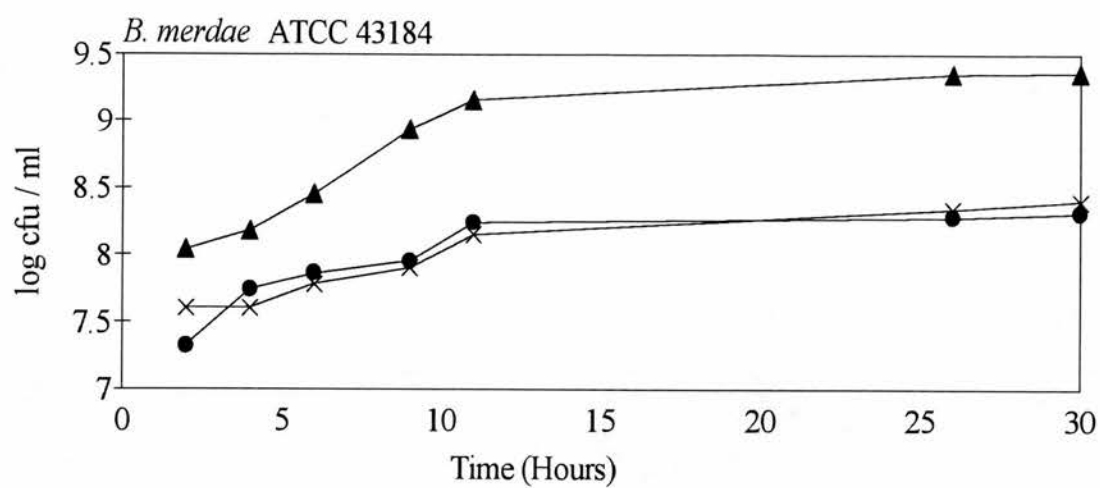


Figure 7 (continued).

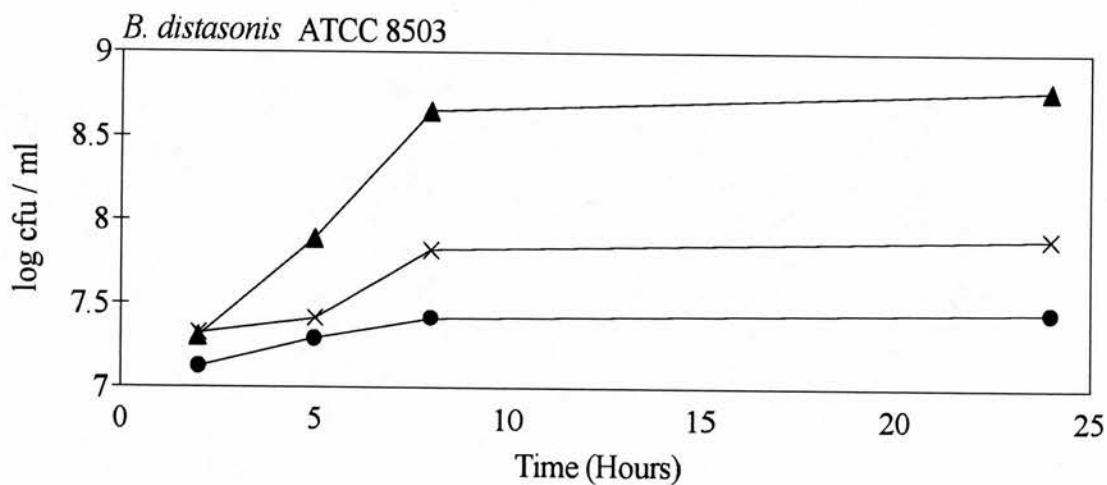
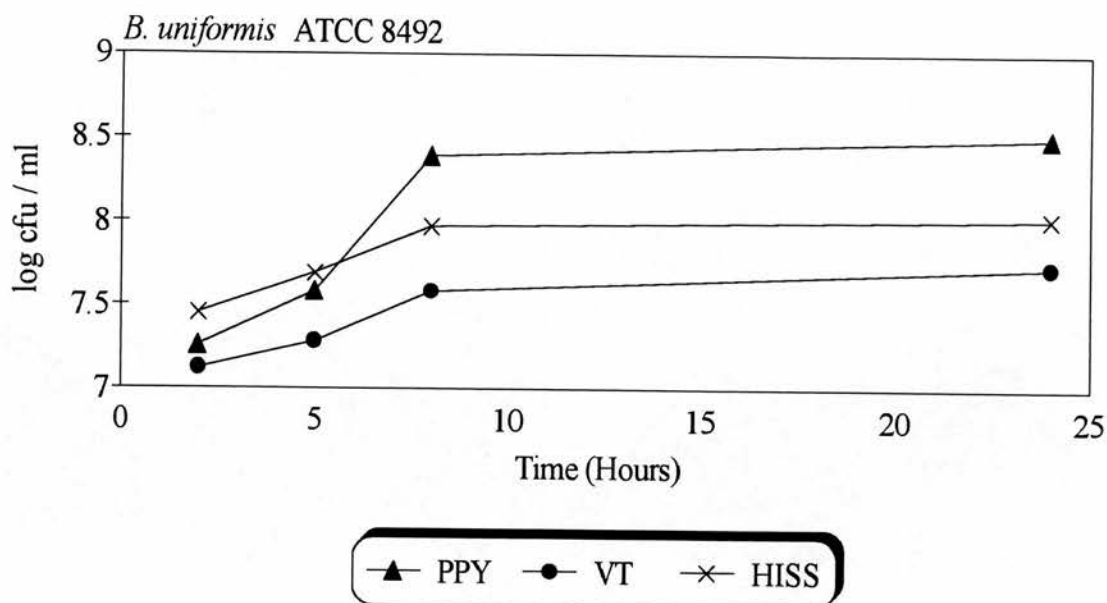


Figure 7 (continued).

Although the density of cells obtained was dependent on growth medium, the different phases of the growth curve were reached at approximately the same time in all media. Logarithmic phase occurred between approximately 2 and 12 hours, and it was decided to harvest all cells at 14 hours when studying cell surface Ags, as this was considered to be early stationary phase.

3.1.2 CAPSULATION OF CELLS IN DIFFERENT MEDIA

A step gradient of Percoll was used to assess the degree of capsulation of 14 *Bacteroides* strains grown to early stationary phase in PPY, VT and HISS. Additionally, selected strains were grown in VT with the addition of various ion chelators, and in a mixture of 50% VT / 50% HISS (VT & S). The results of these experiments are shown in Figures 8a-c: Figure 8a shows a photograph of the Percoll gradients of *B. fragilis* MPRL 1504 following growth in PPY, VT and HISS, and the positions of the LC, SC and NC cells in the gradient are indicated. Figure 8b shows India ink smears of the three cell types of *B. fragilis* MPRL 1504 (LC, SC and NC) which were extracted from the Percoll gradients. Figure 8c shows diagrams of the Percoll gradients of all 14 strains.

Percoll gradients and India ink smears showed all *Bacteroides* strains (except *B. eggerthii* which was non-capsulate) to have a characteristic ratio of cells with large : small : no capsule, and in all cases, this ratio varied with growth medium. There was no inter-species correlation between degree of encapsulation and growth medium; in any given medium some strains were mainly capsulate, whilst others were mainly non-capsulate. Degree of encapsulation in any given medium also varied between different *Bacteroides* strains of the same species.

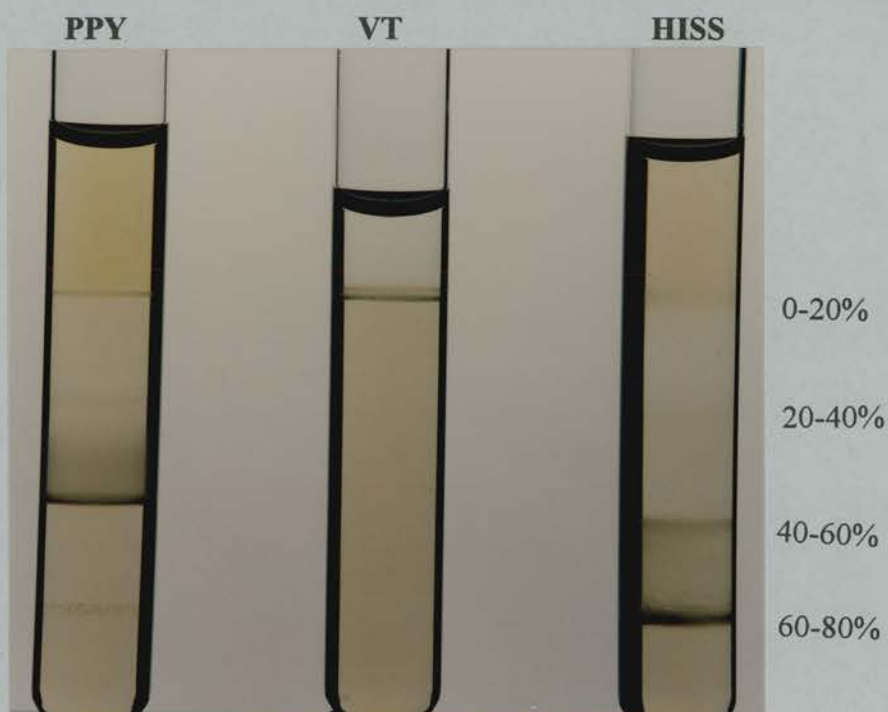


Figure 8a. Four-step (20, 40, 60 and 80%) Percoll density gradients after centrifugation with cultures of *B. fragilis* MPRL 1504 grown to early stationary phase in three media. Cells with large capsules were found at the 0-20% interface, those with small capsules at the 20-40 and 40-60% interfaces, and those with no capsule at the 40-60%, and (mainly) at the 60-80% interface.

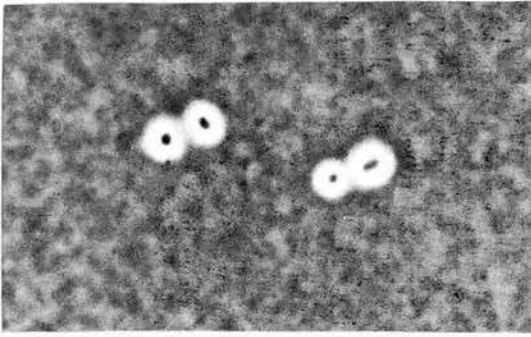
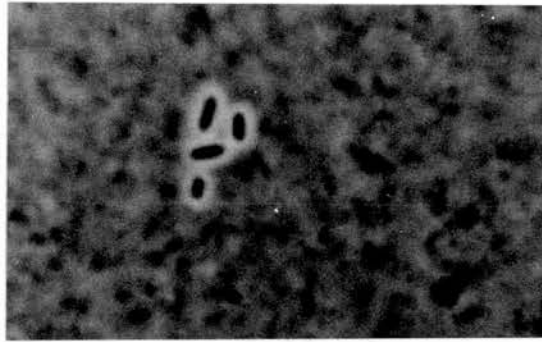
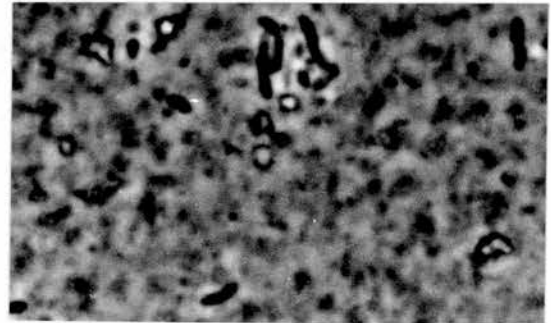
A**B****C**

Figure 8b. Photomicrographs of *B. fragilis* MPRL 1504 showing bacteria with: **A**, large capsules (from VT); **B**, small capsules (from PPY); **C**, no capsules (from HISS). Cells were removed from the most concentrated band in each of the Percoll gradients and stained with India ink.

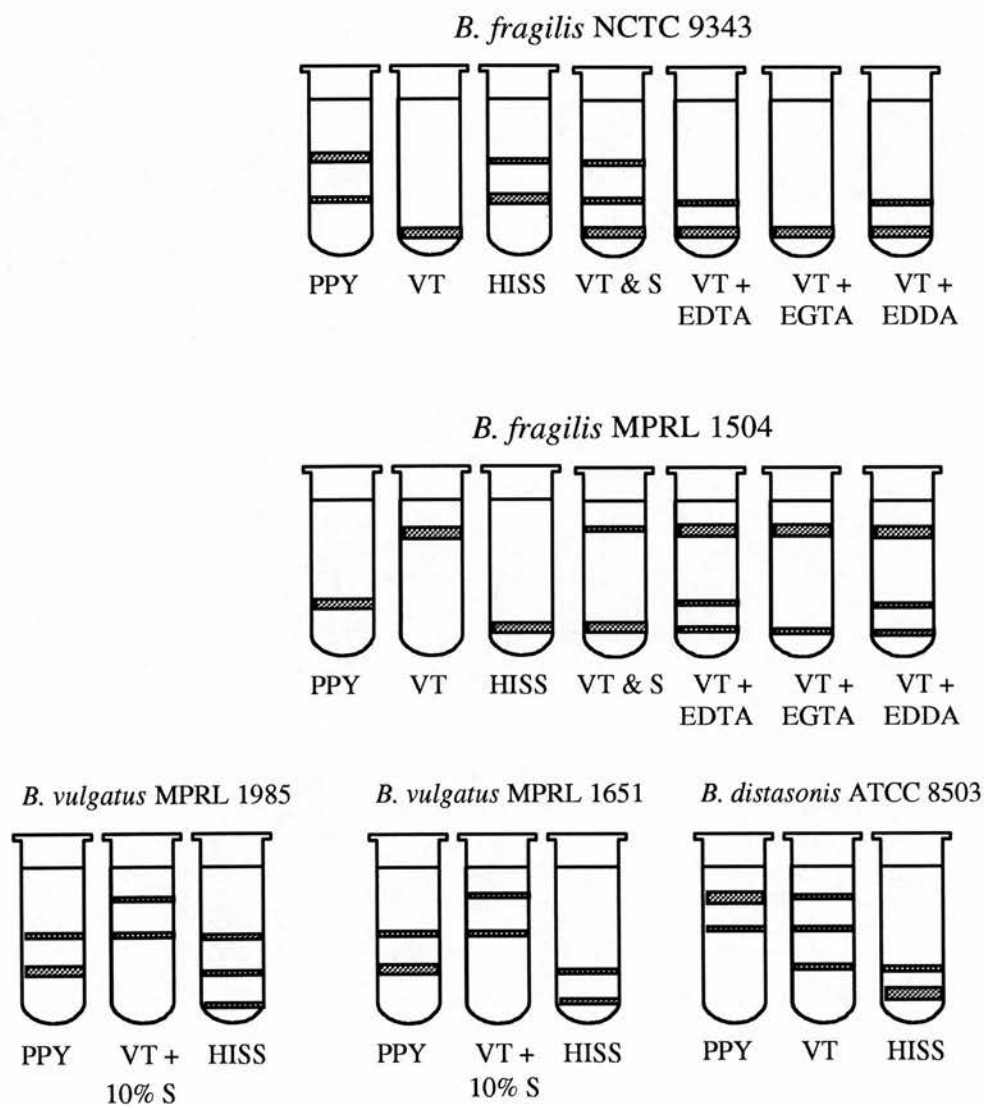
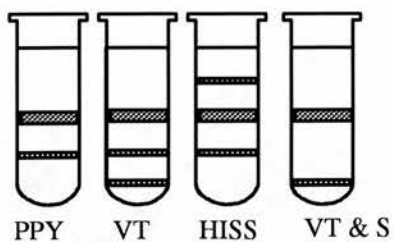
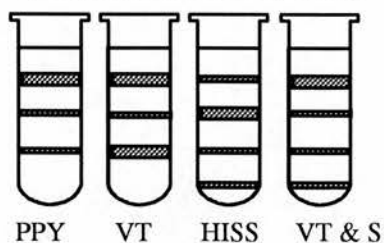


Figure 8c. Diagrammatic representations of the Percoll gradients of 14 *Bacteroides* strains grown in different media. VT & S = 50% VT medium, 50% HISS.

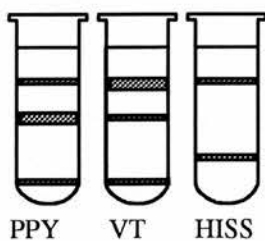
B. thetaiotaomicron NCTC 10582



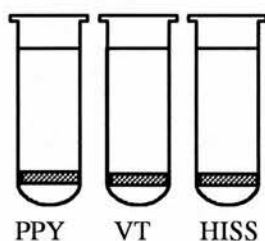
B. thetaiotaomicron MPRL 1959



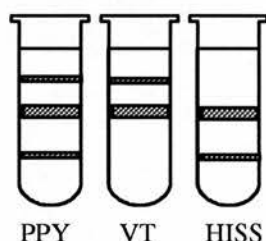
B. ovatus MPRL 2370



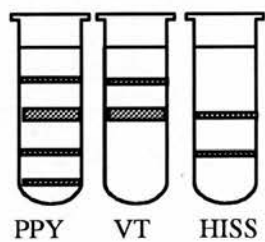
B. eggerthii NCTC 11155



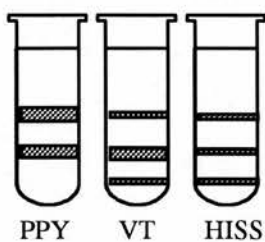
B. caccae MPRL 1555



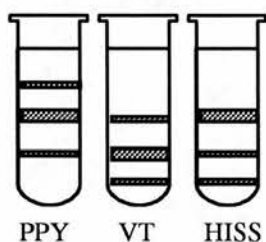
B. variabilis VPI 11368



B. merdae ATCC 43184



B. stercoris ATCC 43183



B. uniformis ATCC 8492

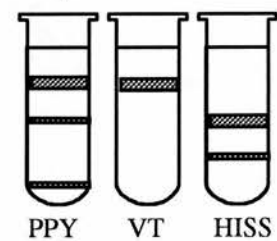


Figure 8c (Continued).

3.1.3 LIPOPOLYSACCHARIDE PROFILES OF *BACTEROIDES* STRAINS GROWN IN THREE MEDIA

The LPS profiles of 25 *Bacteroides* strains grown to early stationary phase in PPY, VT and HISS were examined by PAGE and silver staining. Initially Proteinase K was used to extract the LPS; however, in the majority of cases the resultant LPS stained very weakly with silver, and it appeared that in some cases a large amount of material was not entering the separating gel (results not shown). Therefore, extractions were repeated using the rapid aqueous phenol method of Fomsgaard *et al* (1993), which gave better resolution of the LPS. These gels are shown in Figure 9a-e. Protein molecular weight markers were also electrophoresed with the samples; however, as proteins do not always migrate as a function of their molecular weights, and may not migrate in the same fashion as LPS (Lesse *et al* 1990), any molecular weights of LPS samples quoted are approximate, but are adequate for comparison purposes. Each *Bacteroides* spp. had a species-specific LPS profile, although some inter-strain variations were present. Differences in LPS profiles were apparent in some strains grown in the different media, whereas in other cases there was very little difference observed.

B. vulgatus was the only species which demonstrated a ladder pattern characteristic of smooth LPS. This was also the case following proteinase K extraction (not shown).

There was no obvious ladder pattern in the LPS of the four *B. fragilis* strains tested. The low *Mr* material was similar in all four strains, and all of the strains had the common Ag in all growth media. The largest differences in *B. fragilis* LPS, both between strains and within any given strain grown in the three media, were in the high *Mr* (> 66 kDa) region of the gel. This material may have been CP or slime rather than LPS.

The two *B. thetaiotaomicron* strains had two low *Mr* bands, but in *B. thetaiotaomicron* NCTC 10582 the heavier (~ 16.5 kDa) band was much stronger. *B. ovatus*, *B. uniformis*, *B. variabilis* and *B. caccae* also had two low *Mr* bands, the heavier of which were approximately 16.5 kDa, 15.5 kDa, 12.5 kDa and 19 kDa respectively. In addition, *B. caccae* ATCC 43185 had a third low *Mr* band (~ 15 kDa) when grown in VT and HISS. Overall, the LPS profiles of these five species shared many similarities.

B. distasonis, *B. merdae*, *B. stercoris* and *B. eggerthii* all had one strongly stained low *Mr* band only. *B. eggerthii* MPRL 1216 had a very faint second low *Mr* band (~ 6 kDa) when grown in VT, and in PPY had poorly defined, dark stained material in the medium and high *Mr* regions. *B. merdae* ATCC 43184 also had a second faint low *Mr* band when grown in VT. *B. stercoris* had a larger amount of low *Mr* material when grown in HISS compared to when grown in PPY or VT. Overall, the LPS profiles of these four species were quite similar.

Although the samples examined in these experiments were pure aqueous phenol extracts, it was suspected that they were not pure LPS; the bands present in the smooth LPS region in some of the *B. fragilis* strains were not evenly spaced and did not look like smooth LPS. Also, there was a lot of dark staining, diffuse material in some tracks, particularly in the high *Mr* regions, and in many cases material was staining in the stacking gel which had never entered the separating gel.

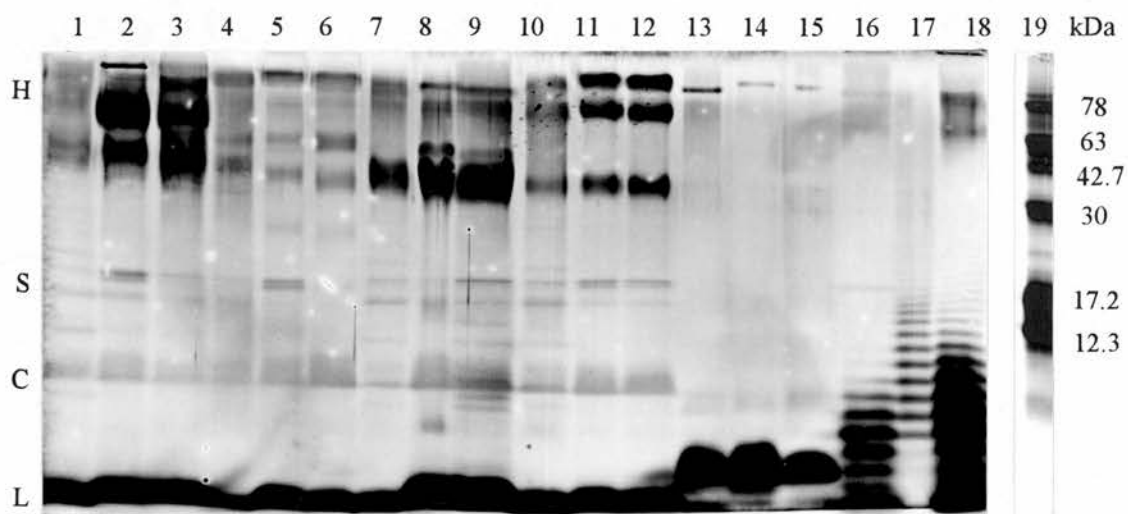
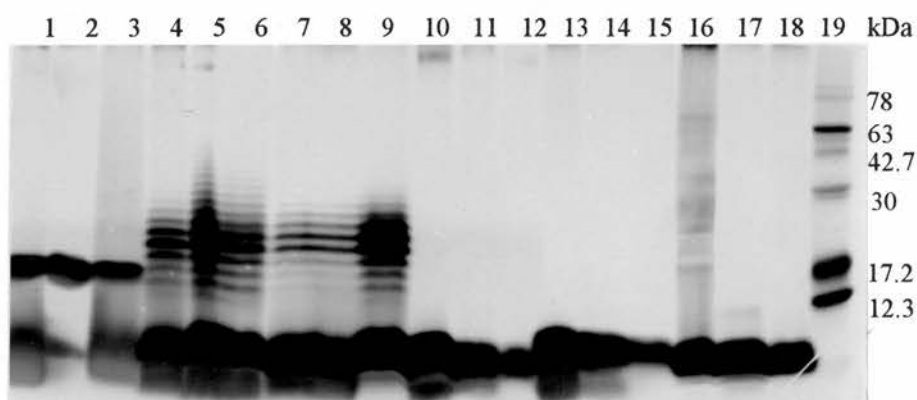
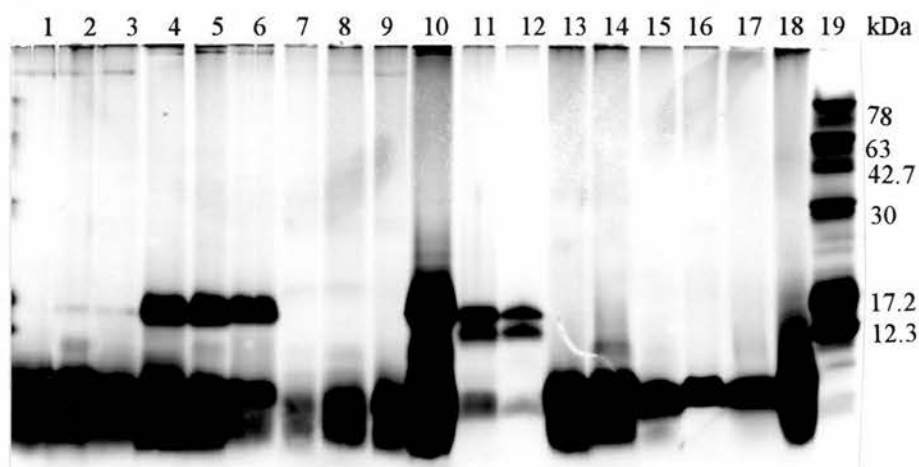


Figure 9. Silver-stained (14% acrylamide) LPS profiles of rapid aqueous phenol extracts of 25 *Bacteroides* strains following growth to early stationary phase in PPY, VT and HISS.

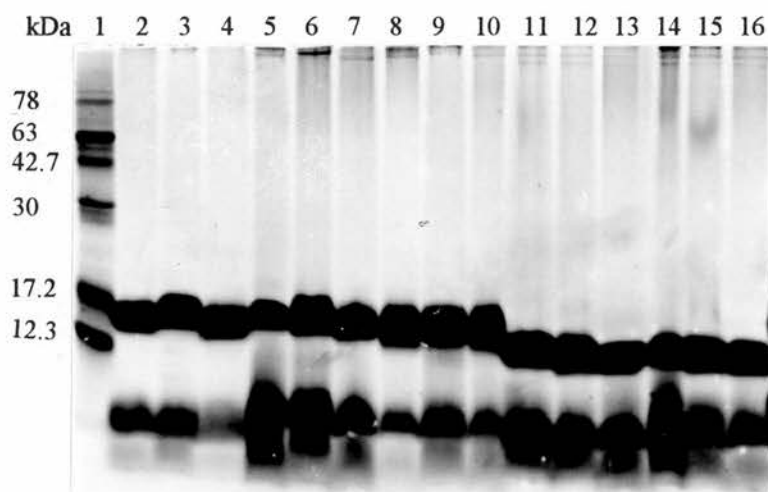
9a. Tracks: **1**, *B. fragilis* NCTC 9343 (PPY); **2**, *B. fragilis* NCTC 9343 (VT); **3**, *B. fragilis* NCTC 9343 (HISS); **4**, *B. fragilis* MPRL 1504 (PPY); **5**, *B. fragilis* MPRL 1504 (VT); **6**, *B. fragilis* MPRL 1504 (HISS); **7**, *B. fragilis* MPRL 1652 (PPY); **8**, *B. fragilis* MPRL 1652 (VT); **9**, *B. fragilis* MPRL 1652 (HISS); **10**, *B. fragilis* MPRL 1978 (PPY); **11**, *B. fragilis* MPRL 1978 (VT); **12**, *B. fragilis* MPRL 1978 (HISS); **13**, *B. thetaiotaomicron* MPRL 1959 (PPY); **14**, *B. thetaiotaomicron* MPRL 1959 (VT); **15**, *B. thetaiotaomicron* MPRL 1959 (HISS); **16**, *B. vulgatus* MPRL 1651 (PPY); **17**, *B. vulgatus* MPRL 1651 (VT); **18**, *B. vulgatus* MPRL 1651 (HISS); **19**, *Mr* markers. Abbreviations: L = low *Mr* material (lipid A and core), C = *B. fragilis* common Ag, S = smooth LPS region, H = high *Mr* material (possibly CP or slime).



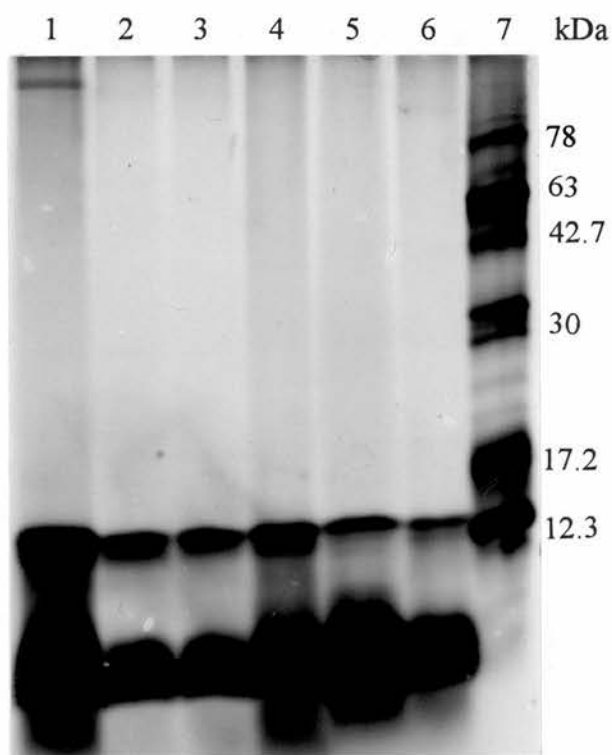
9b. Tracks: **1**, *B. thetaiotaomicron* NCTC 10582 (PPY); **2**, *B. thetaiotaomicron* NCTC 10582 (VT); **3**, *B. thetaiotaomicron* NCTC 10582 (HISS); **4**, *B. vulgatus* MPRL 1985 (PPY); **5**, *B. vulgatus* MPRL 1985 (VT); **6**, *B. vulgatus* MPRL 1985 (HISS); **7**, *B. vulgatus* NCTC 11154 (PPY); **8**, *B. vulgatus* NCTC 11154 (VT); **9**, *B. vulgatus* NCTC 11154 (HISS); **10**, *B. eggerthii* NCTC 11155 (PPY); **11**, *B. eggerthii* NCTC 11155 (VT); **12**, *B. eggerthii* NCTC 11155 (HISS); **13**, *B. eggerthii* MPRL 1523 (PPY); **14**, *B. eggerthii* MPRL 1523 (VT); **15**, *B. eggerthii* MPRL 1523 (HISS); **16**, *B. eggerthii* MPRL 1216 (PPY); **17**, *B. eggerthii* MPRL 1216 (VT); **18**, *B. eggerthii* MPRL 1216 (HISS); **19**, Mr markers.



9c. Tracks: **1**, *B. distasonis* MPRL 1522; **2**, *B. distasonis* MPRL 1522 (VT); **3**, *B. distasonis* MPRL 1522 (HISS); **4**, *B. caccae* MPRL 1555 (PPY); **5**, *B. caccae* MPRL 1555 (VT); **6**, *B. caccae* MPRL 1555 (HISS); **7**, *B. distasonis* ATCC 8503 (PPY); **8**, *B. distasonis* ATCC 8503 (VT); **9**, *B. distasonis* ATCC 8503 (HISS); **10**, *B. caccae* ATCC 43185 (PPY); **11**, *B. caccae* ATCC 43185 (VT); **12**, *B. caccae* ATCC 43185 (HISS); **13**, *B. merdae* ATCC 43184 (PPY); **14**, *B. merdae* ATCC 43184 (VT); **15**, *B. merdae* ATCC 43184 (HISS); **16**, *B. stercoris* ATCC 43183 (PPY); **17**, *B. stercoris* ATCC 43183 (VT); **18**, *B. stercoris* ATCC 43183 (HISS); **19**, *Mr* markers.



9d. Tracks: **1**, *Mr* markers; **2**, *B. ovatus* ATCC 8483 (PPY); **3**, *B. ovatus* ATCC 8483 (VT); **4**, *B. ovatus* ATCC 8483 (HISS); **5**, *B. ovatus* MPRL 1987 (PPY); **6**, *B. ovatus* MPRL 1987; **7**, *B. ovatus* MPRL 1987 (HISS); **8**, *B. ovatus* MPRL 2370 (PPY); **9**, *B. ovatus* MPRL 2370 (VT); **10**, *B. ovatus* MPRL 2370 (HISS); **11**, *B. variabilis* VPI 11368 (PPY); **12**, *B. variabilis* VPI 11368 (VT); **13**, *B. variabilis* VPI 11368 (HISS); **14**, *B. variabilis* MPRL 2244 (PPY); **15**, *B. variabilis* MPRL 2244 (VT); **16**, *B. variabilis* MPRL 2244 (HISS).



9e. Tracks: **1**, *B. uniformis* ATCC 8492 (PPY); **2**, *B. uniformis* ATCC 8492 (VT); **3**, *B. uniformis* ATCC 8492 (HISS); **4**, *B. uniformis* MPRL 1542 (PPY); **5**, *B. uniformis* MPRL 1542 (VT); **6**, *B. uniformis* MPRL 1542 (HISS); **7**, *Mr* markers.

3.1.4 COLONY MORPHOLOGY AND LIPOPOLYSACCHARIDE PROFILES OF DIFFERENT CELL SUB-POPULATIONS FROM PERCOLL GRADIENTS

To look at differences in sub-populations of cells in selected strains, bacteria were grown in VT (or VT + 10% HISS for *B. vulgatus*) and centrifuged in Percoll gradients as before. Sub-populations were then removed from the gradients, grown in fresh medium and streaked onto CBA plates. Following growth to early stationary phase in liquid medium, Percoll centrifugation was repeated and Proteinase K extractions were carried out. Plates were incubated for 72 h before colony morphology was examined.

No major differences were observed in any of the strain sub-populations in colony morphology following growth on CBA. When colonies were inoculated into fresh VT or VT + 10% HISS and centrifuged in Percoll as before, the cells had reverted to the phenotypes seen in Figure 8c.

Capsulate cells remained capsulate after sub-culture. In most cases, in LC populations, some SC cells had appeared following sub-culture (although the majority were still of the LC phenotype), and the reverse was true for SC populations. NC cells had reverted to a mixture of capsulate and NC cells following sub-culture, although the majority were still NC (results not shown). Bearing this in mind, the LPS profiles of the different sub-populations are shown in Figure 10a-b. There were differences in the LPS profiles of some strain sub-populations, both in the low and high *Mr* regions. Of particular interest is *B. ovatus* MPRL 2370, which had an extra low *Mr* band in the NC sub-population, and the two *B. fragilis* strains, which showed differences in the high and low *Mr* regions in different sub-populations. *B. vulgatus* was the only species with an obvious ladder pattern.

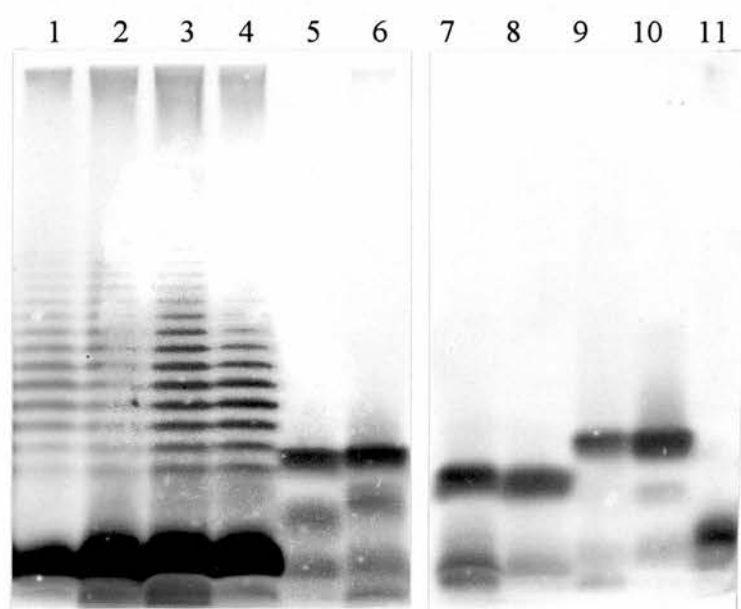


Figure 10a. Silver-stained PAGE (14% acrylamide) LPS profiles of Proteinase K extracts of sub-populations of 11 *Bacteroides* strains grown to early stationary phase in VT. Tracks: **1**, *B. vulgatus* MPRL 1985 (LC); **2**, *B. vulgatus* MPRL 1985 (SC); **3**, *B. vulgatus* MPRL 1651 (LC); **4**, *B. vulgatus* MPRL 1651 (SC); **5**, *B. caccae* MPRL 1555 (LC); **6**, *B. caccae* MPRL 1555 (SC); **7**, *B. variabilis* VPI 11368 (LC); **8**, *B. variabilis* VPI 11368 (SC); **9**, *B. ovatus* MPRL 2370 (LC); **10**, *B. ovatus* MPRL 2370 (NC); **11**, *B. eggerthii* NCTC 11155 (NC).

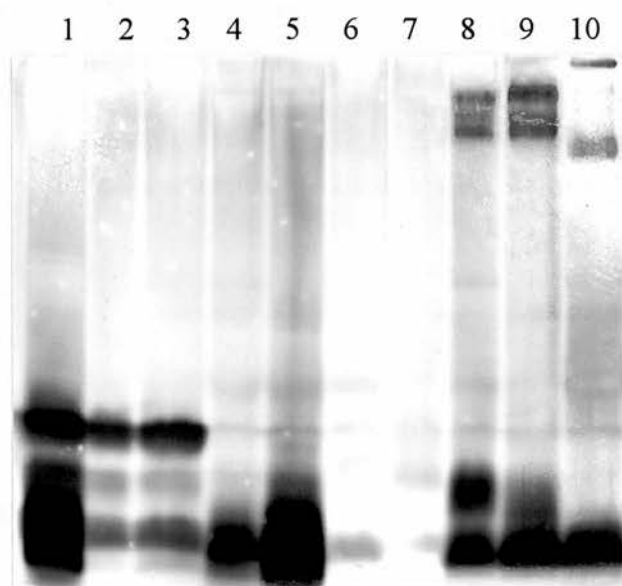


Figure 10b. Tracks: **1**, *B. thetaiotaomicron* NCTC 10582 (SC: 20-40% interface); **2**, *B. thetaiotaomicron* NCTC 10582 (SC: 40-60% interface); **3**, *B. thetaiotaomicron* (NC); **4**, *B. distasonis* ATCC 8503 (SC); **5**, *B. distasonis* ATCC 8503 (NC); **6**, *B. uniformis* ATCC 8492 (LC); **7**, *B. uniformis* ATCC 8492 (SC); **8**, *B. fragilis* NCTC 9343 (SC); **9**, *B. fragilis* NCTC 9343 (NC); **10**, *B. fragilis* MPRL 1504 (LC).

3.2 BACTERICIDAL ACTIVITY OF HUMAN SERUM AGAINST BACTEROIDES SPECIES

Resistance to the bactericidal effects of serum complement is a well-recognised virulence determinant, but to date, work carried out on complement resistance of *Bacteroides* spp. has been very limited. The purpose of these experiments was therefore i) to investigate whether different growth environments affected the sensitivity of *Bacteroides* spp. to serum, ii) to investigate the mechanism of complement resistance in *Bacteroides* spp., iii) to investigate whether different mechanisms of processing serum affected its bactericidal capabilities, iv) to investigate classical and alternative pathway activation of complement by *Bacteroides* strains and v) to compare the bactericidal capability of serum from healthy individuals with that from SIRS patients.

3.2.1 COMPARISON OF TWO METHODS OF SERUM PROCESSING

Serum from five healthy volunteers was processed by two different methods termed A and B (described in Section 2.1.2). It was then tested for its ability to kill *B. fragilis* NCTC 9343, *B. fragilis* MPRL 1504 and *B. vulgatus* MPRL 1985 grown in PPY (Figure 11). The individual and pooled serum samples collected by the two different methods were also examined by a sheep erythrocyte lysis assay to determine the haemolytic complement value or CH50 (Table 3). In this assay, a normal CH50 value was within the range 50 to 150 CH50 units / ml.

With all three strains, serum processed by method A was more bactericidal than that processed by method B. In addition, the CH50 values of the individual and pooled sera were greater in serum processed by method A, indicating that complement activity was better preserved with this method; in three individuals, and in the pooled serum, CH50 values had decreased to below normal values when processed by method B. Therefore, in all subsequent experiments, serum processed by method A

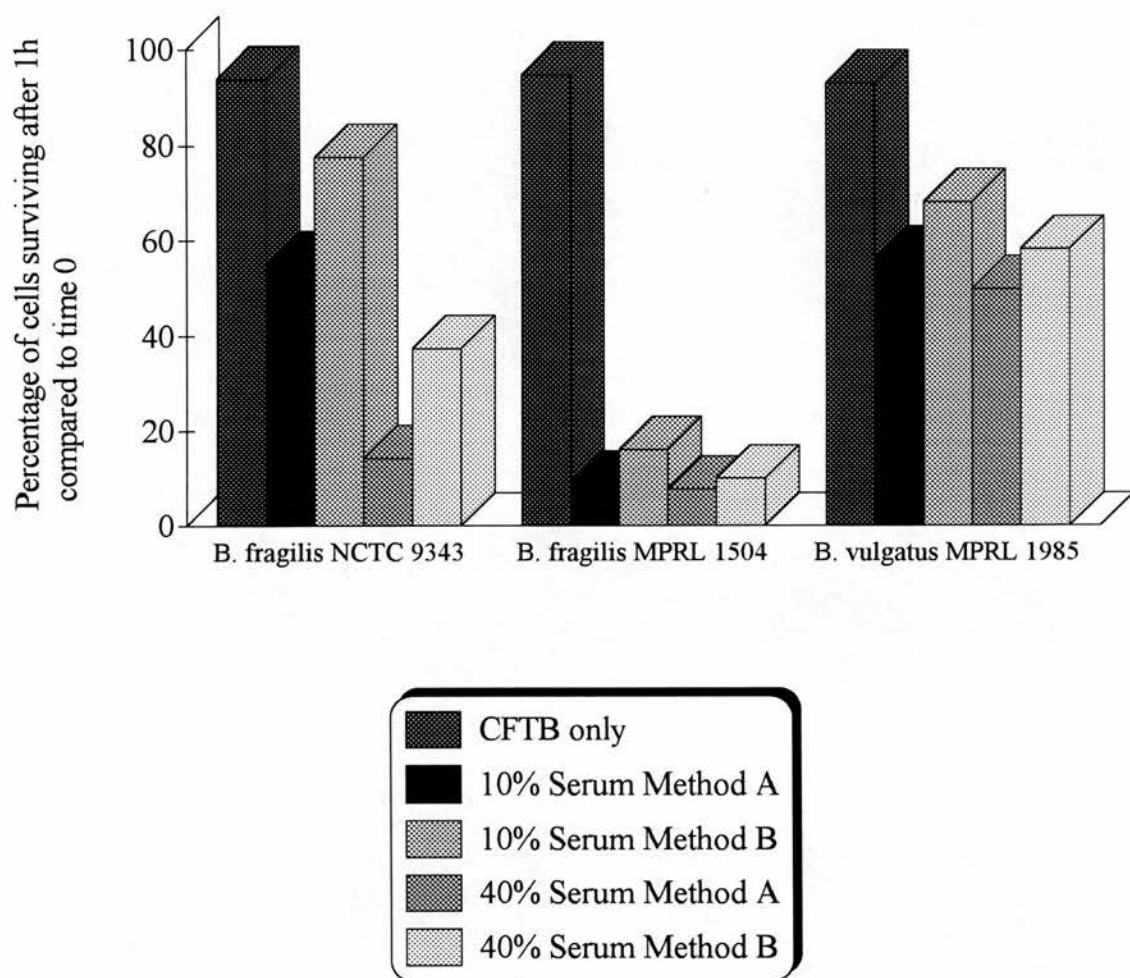


Figure 11. Bactericidal activity of pooled serum from healthy volunteers processed by two different methods. Results shown are mean percentages calculated from at least two replicates.

was used.

Table 3. CH50 values of individual and pooled serum from healthy volunteers processed by two different methods.

	CH50 (units / ml) Method A	CH50 (units / ml) Method B
Volunteer 1	56.2	48.9
Volunteer 2	123.0	88.6
Volunteer 3	83.2	71.9
Volunteer 4	49.9	32.5
Volunteer 5	59.1	39.5
Pooled serum (all volunteers)	63.1	49.1

3.2.2 THE INFLUENCE OF GROWTH MEDIUM ON SERUM SENSITIVITY OF *BACTEROIDES* SPECIES

To investigate whether growth environment affected the sensitivity of *Bacteroides* spp. to complement, 12 strains (some clinical, some faecal isolates) were grown in PPY, VT (or VT + 10% HISS for *B. vulgatus*) and HISS, and then tested for their ability to resist complement. When grown in PPY, all strains were, to varying degrees, sensitive to complement. However, when grown in VT, six of the strains (*B. fragilis* NCTC 9343, *B. fragilis* MPRL 1504, *B. caccae* MPRL 1555, *B. thetaiotaomicron* MPRL 1959, *B. ovatus* MPRL 2370 and *B. vulgatus* MPRL 1985) became markedly more resistant to complement. With the exception of *B. vulgatus* MPRL 1985, these strains became totally resistant to complement when grown in HISS. In every case, survival in CFTB alone was between 90 and 100%. Heat inactivation of serum destroyed bactericidal activity in every case where a strain was killed by active serum. Figure 12a-b shows two examples of strains tested, one of which (*B. fragilis* MPRL 1504) showed changes in complement

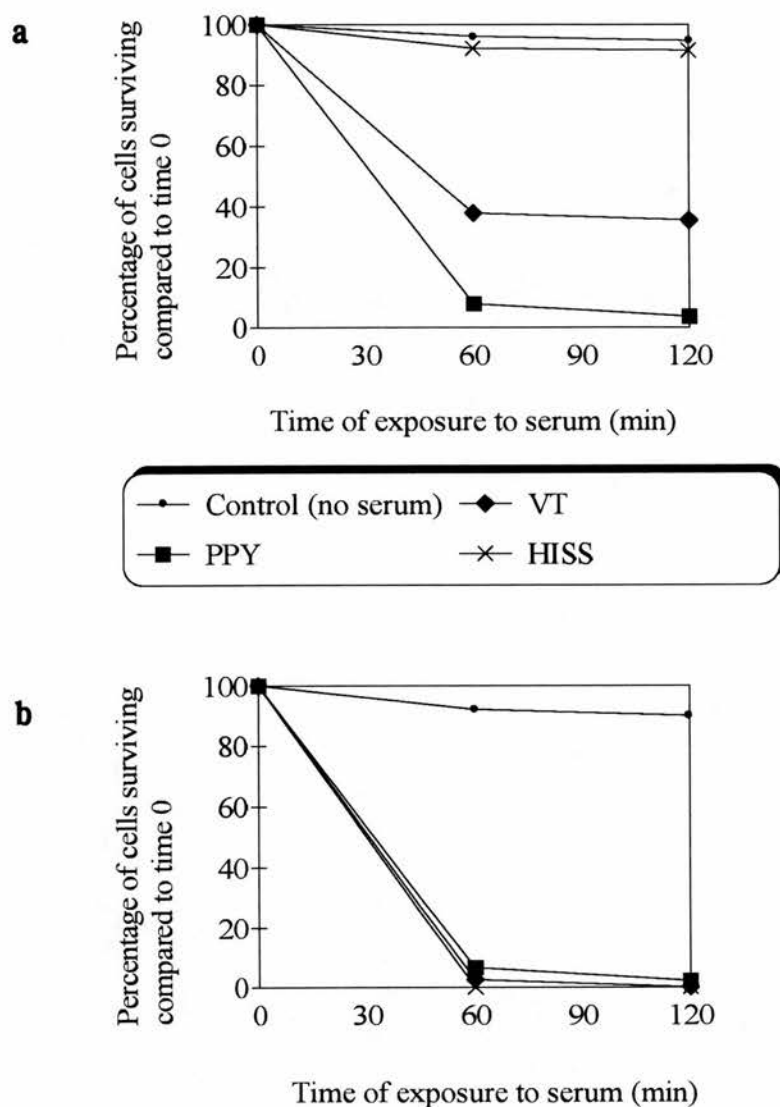


Figure 12. Survival of **a:** *B. fragilis* MPRL 1504 and **b:** *B. distasonis* ATCC 8503 in 40% pooled human serum following growth in three media. Points show mean percentage survival calculated from four replicates.

resistance when grown in the different media, and the other of which (*B. distasonis* ATCC 8503) remained sensitive to complement in the different media. Table 4 shows the results for all 12 strains incubated with 40% serum.

Table 4. Survival of 12 *Bacteroides* strains after 1 h in 40% human serum following growth in three media. Figures shown are mean percentage survival of bacteria calculated from at least two replicates.

Percent Survival of Bacteria Grown in			
Strain	PPY	VT	HISS
<i>B. fragilis</i> MPRL 1504	7.8	37.9	92.2
<i>B. fragilis</i> NCTC 9343	14.0	68.1	94.2
<i>B. caccae</i> MPRL 1555	10.2	48.9	91.3
<i>B. ovatus</i> MPRL 2370	74.4	85.6	98.1
<i>B. thetaiotaomicron</i> MPRL 1959	52.9	71.8	97.6
<i>B. thetaiotaomicron</i> NCTC 10582	23.3	19.7	25.1
<i>B. uniformis</i> ATCC 8492	13.0	11.3	10.6
<i>B. vulgatus</i> MPRL 1985	49.0	61.1	41.5
<i>B. vulgatus</i> MPRL 1651	14.4	21.5	15.5
<i>B. eggerthii</i> NCTC 11155	0.0	3.2	0.0
<i>B. distasonis</i> ATCC 8503	6.7	0.0	2.6
<i>B. variabilis</i> VPI 11368	0.0	0.0	1.2

3.2.3 INVESTIGATION OF THE MECHANISM OF COMPLEMENT RESISTANCE IN *BACTEROIDES FRAGILIS*

To investigate the biochemical basis for the observed changes in complement resistance of certain strains when grown in different media, the two *B. fragilis* strains (NCTC 9343 and MPRL 1504) which demonstrated these differences were used.

Expression of cell surface Ags and binding of C3b to the cell surface were investigated in the three media. As cells had to be grown in 1 L cultures for extraction of cell surface Ags, instead of using HISS alone, a mixture of 50% HISS / 50% VT (VT & S) was used; cells grown in this were still totally resistant to complement (results not shown).

3.2.3a Capsulation of Cells in Different Media

Differences in cell capsulation in different media, both between different strains grown in the same media, and within any given strain grown in different media, have already been demonstrated (Figure 8c).

3.2.3b Outer Membrane Protein Expression in Different Media

Sarkosyl extracts of cells grown in the three media were visualised by PAGE followed by Coomassie blue staining (Figure 13) and immunoblotting (Figure 14a-b). In both strains, the protein concentration of the extracts from cells grown in VT was too weak for the proteins to stain with Coomassie blue. However, several differences in OMP expression were seen between cells grown in PPY and VT & S by Coomassie blue staining. The OMP profiles of the two strains were similar, and varied in a similar manner according to growth medium. Both strains had six bands corresponding to proteins of approximately 72, 39, 34, 25, 16 and 10 kDa which stained stronger following growth in PPY than in VT & S. Both strains grown in VT & S had three proteins of approximately 91, 64 and 16.5 kDa which stained more heavily than in PPY. Immunoblots of the OMPs using hyperimmune rabbit serum gave similar profiles to those seen with Coomassie blue, except the OMPs from the VT-grown cells stained stronger (although not strongly enough to be able to make adequate comparisons), and proteins of *Mr* less than approximately 35 kDa stained very poorly.

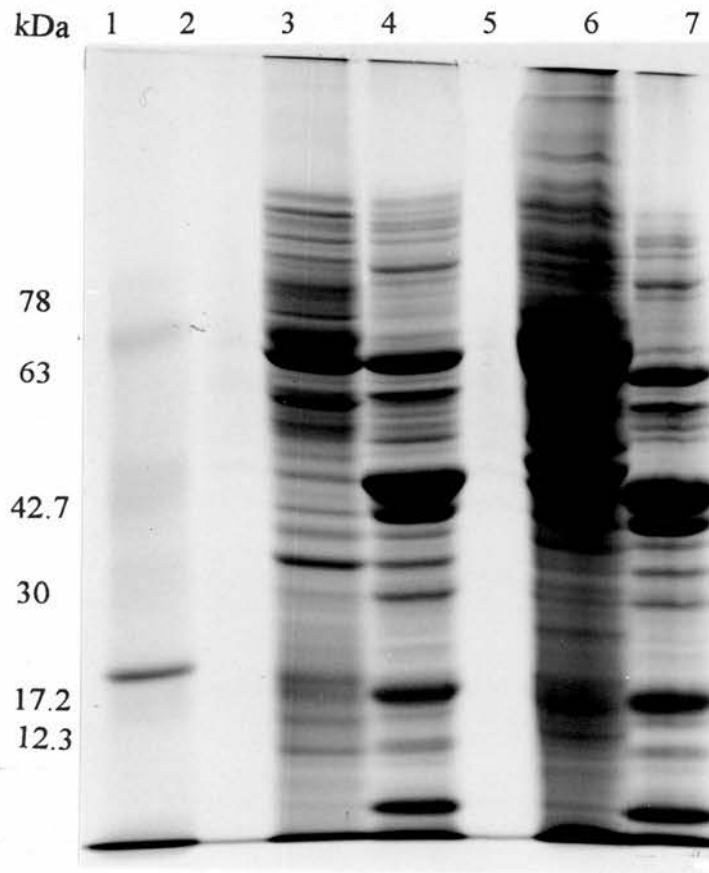


Figure 13. Coomassie blue-stained SDS-PAGE (10% acrylamide) OMP profiles of sarkosyl extracts of *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 following growth to early stationary phase in PPY, VT and VT & S. Tracks: 1, *Mr* markers; 2, MPRL 1504 (VT); 3, MPRL 1504 (VT & S); 4, MPRL 1504 (PPY); 5, NCTC 9343 (VT); 6, NCTC 9343 (VT & S); 7, NCTC 9343 (PPY). Amounts of protein added per track were: tracks 3, 4, 6 and 7 - 40 μ g; track 2 - 7 μ g; track 5 - 14 μ g.

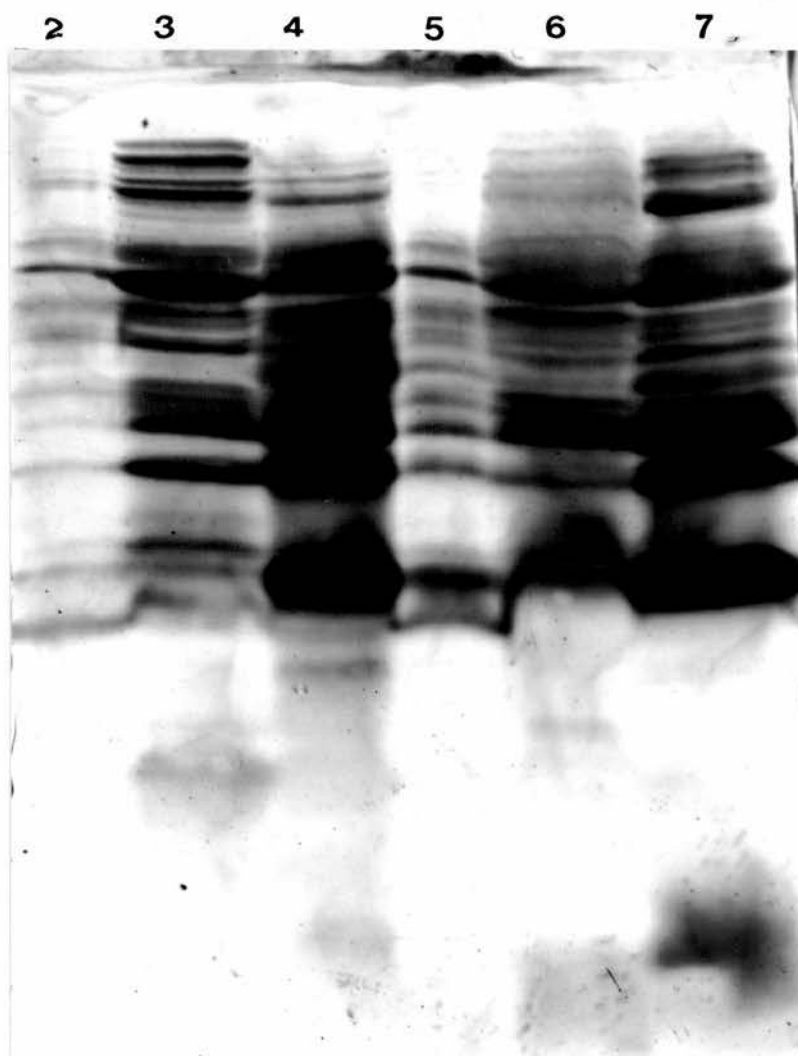


Figure 14a. Immunoblot of SDS-polyacrylamide (10%) gel of OMPs from *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 following growth to early stationary phase in PPY, VT and VT & S, using hyperimmune polyclonal rabbit serum (raised against the OMPs of *B. fragilis* NCTC 9344). Tracks and protein concentrations as in Figure 13.

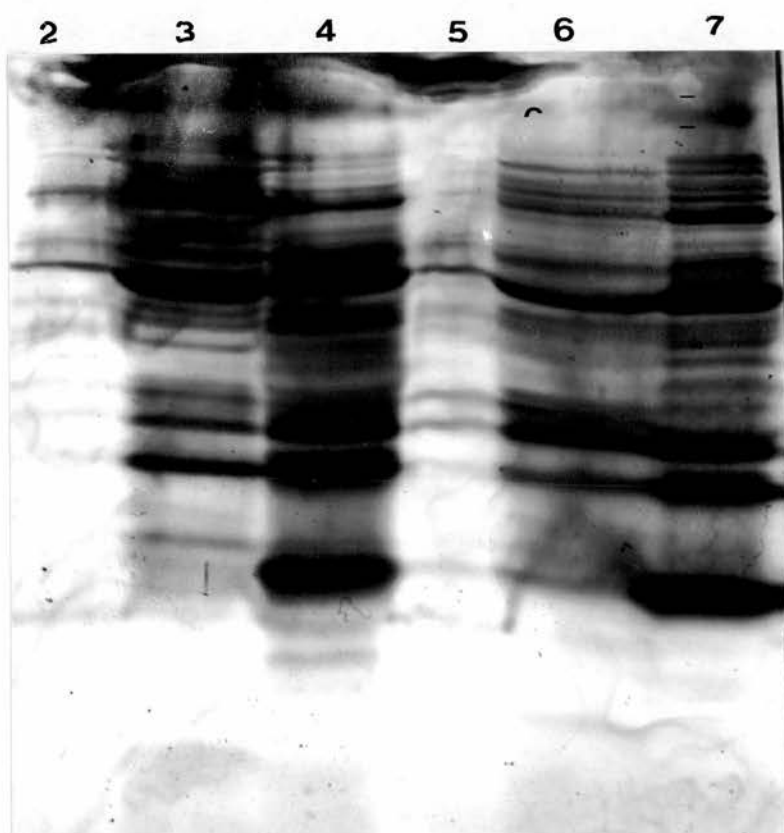


Figure 14b. Immunoblot of SDS-polyacrylamide (10%) gel of OMPs from *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 following growth to early stationary phase in PPY, VT and VT & S, using hyperimmune polyclonal rabbit serum (raised against *B. fragilis* MPRL 1504 whole cells). Tracks and protein concentrations as in Figure 13.

In a previous study, Patrick & Lutton (1990) found that *B. fragilis* NCTC 9343 grown *in vivo* appeared to express proteins not seen when grown *in vitro*; these proteins were subsequently shown to be mouse albumin and IgG, and could be removed by washing. In the present study, cells were washed prior to extracting OMPs, which should have removed any media-derived associated proteins. However, as a further control, to examine whether any of the bands could be attributed to the growth media, PPY alone and VT & S alone were electrophoresed and stained with Coomassie blue and silver. Apart from the dye front, no bands were seen (results not shown).

3.2.3c Lipopolysaccharide Profiles of Cells Grown in Different Media

Aqueous phenol extracts of cells (with the final ultracentrifugation step omitted), were examined by PAGE, followed by silver staining and immunoblotting. The silver-stained PAGE profiles are shown in Figure 15. The staining of these aqueous phenol extracts was stronger than that of the rapid phenol extracts in Figure 9a, probably reflecting differences in the concentration of material added to the tracks. However, a similar pattern of staining was observed in the full-scale aqueous phenol extracts; low and high *Mr*-staining material and the common Ag were present in both strains in all media, but there was no evidence of an evenly-spaced ladder pattern characteristic of smooth LPS. Some bands were present in the smooth LPS region, especially in VT & S-grown bacteria, but they did not look like the classical ladder pattern of smooth LPS, and may have been contaminating protein. The biggest differences in the silver-stained aqueous phenol extracts were in the high *Mr* material, which may have not been LPS.

Immunoblotting of the aqueous phenol extracts using hyperimmune rabbit serum (Figure 16) revealed differences not observed with silver staining. Low *Mr* material (below the common Ag) was poorly stained, as was also observed in immunoblots of OMPs. *B. fragilis* NCTC 9343 extracts showed no evidence of a ladder pattern after

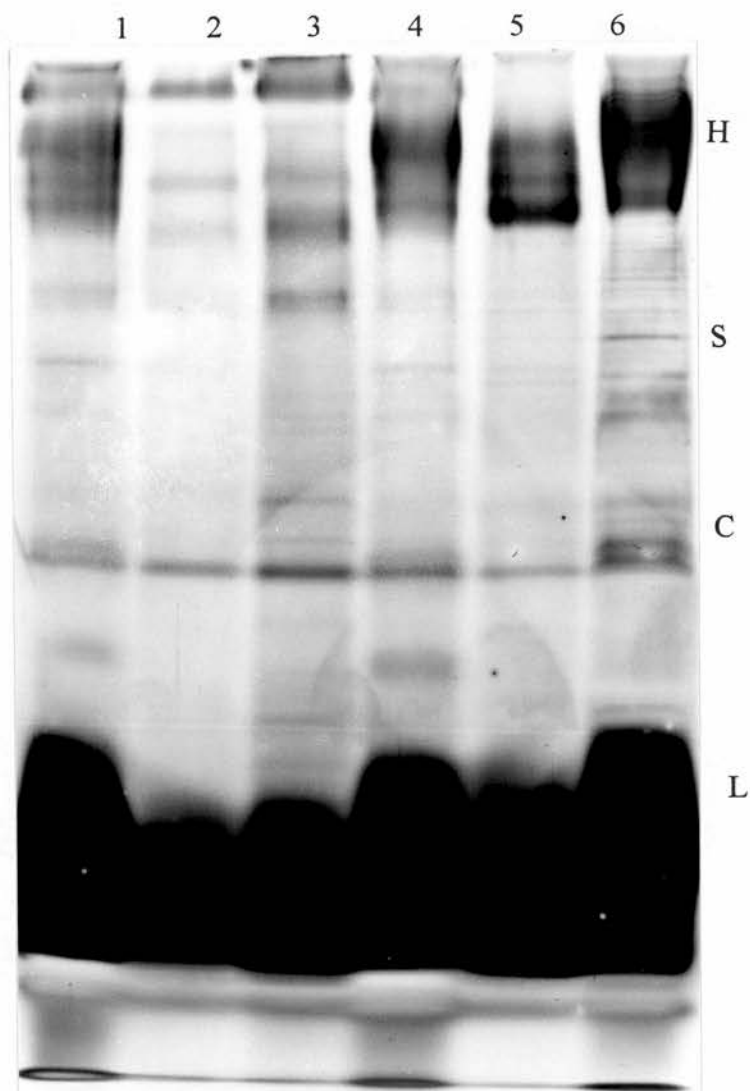


Figure 15. Silver-stained PAGE (14% acrylamide) profiles of aqueous phenol extracts (100 μg / track) of *B. fragilis* MPRL 1504 and *B. fragilis* NCTC 9343 following growth to early stationary phase in PPY, VT and VT & S. Tracks: 1, MPRL 1504 (PPY); 2, MPRL 1504 (VT); 3, MPRL 1504 (VT & S); 4, NCTC 9343 (PPY); 5, NCTC 9343 (VT); 6, NCTC 9343 (VT & S). Abbreviations as in Figure 9a.

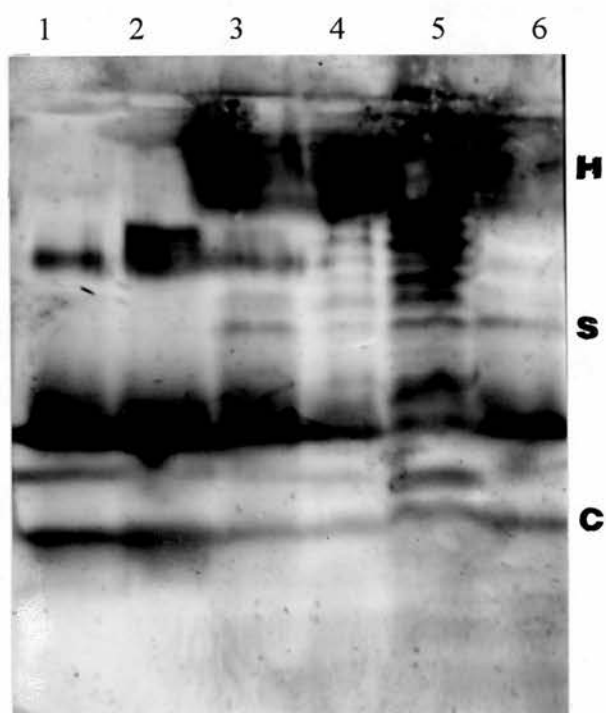


Figure 16. Immunoblot of polyacrylamide (14%) gel of aqueous phenol extracts (200 μ g / track) of *B. fragilis* MPRL 1504 and *B. fragilis* NCTC 9343 following growth to early stationary phase in PPY, VT and VT & S, using hyperimmune rabbit serum raised against *B. fragilis* NCTC 9344 whole cells. Tracks: **1**, MPRL 1504 (VT); **2**, MPRL 1504 (VT & S); **3**, MPRL 1504 (PPY); **4**, NCTC 9343 (VT); **5**, NCTC 9343 (VT & S); **6**, NCTC 9343 (PPY). Abbreviations as in Figure 9a.

growth in PPY (where they were sensitive to complement). However, after growth in VT (where they were intermediately resistant to complement), a weak ladder pattern was evident, and after growth in VT & S (where they were totally resistant to complement) a strong ladder pattern was evident. A ladder pattern was not present in *B. fragilis* MPRL 1504, although some dark stained material was present in the smooth LPS region, which increased in intensity from PPY to VT to VT & S, as the ladder pattern did in *B. fragilis* NCTC 9343.

3.2.3d Binding of C3b to the Cell Surface

Binding of complement component C3b to the bacterial cell surface is a crucial initial step in the sequence leading to cell lysis. To see whether this occurred equally to bacteria grown in different media, whole bacterial cells were coated onto microtitre plates, and binding of C3b to the cells was assessed by ELISA. The results of this are shown in Figure 17. C3b bound to both strains grown in all media. Although there were differences in OD (and therefore the amount of C3b bound) within each given strain, these were relatively small, and when the standard errors were taken into consideration, there was no significant difference in binding of C3b to the cells of either strain when grown in different media. The amount of C3b bound to *B. fragilis* MPRL 1504 was slightly greater than that bound to *B. fragilis* NCTC 9343, but this may have reflected small differences in numbers of cells coated onto plates.

3.2.3e Complement Resistance of Cells Grown in VT & S then PPY

In order to see whether cells grown in VT & S maintained their ability to resist complement, bacteria were grown initially in VT & S, and then a 1% inoculum of the resulting culture was placed into PPY. Following growth, the cells were assayed for complement resistance, a further 1% inoculum of cells was added to fresh PPY and the above process was repeated. As a control, the same strains were grown initially in PPY then treated as above. The result of this experiment is shown in Figure 18. In

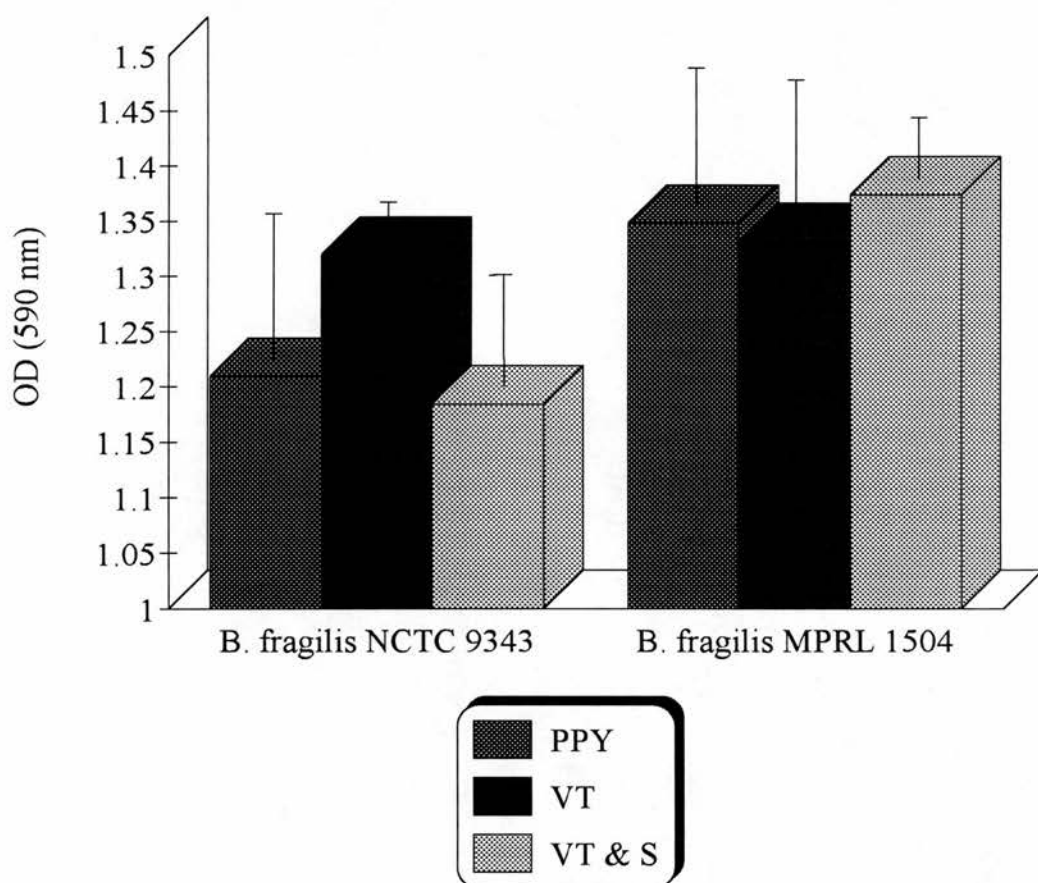


Figure 17. Measurement of binding of C3b to whole cells of *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 in ELISA. Cells were grown to early stationary phase in three media. Results shown are average ODs from four replicates. \bar{T} = standard error.

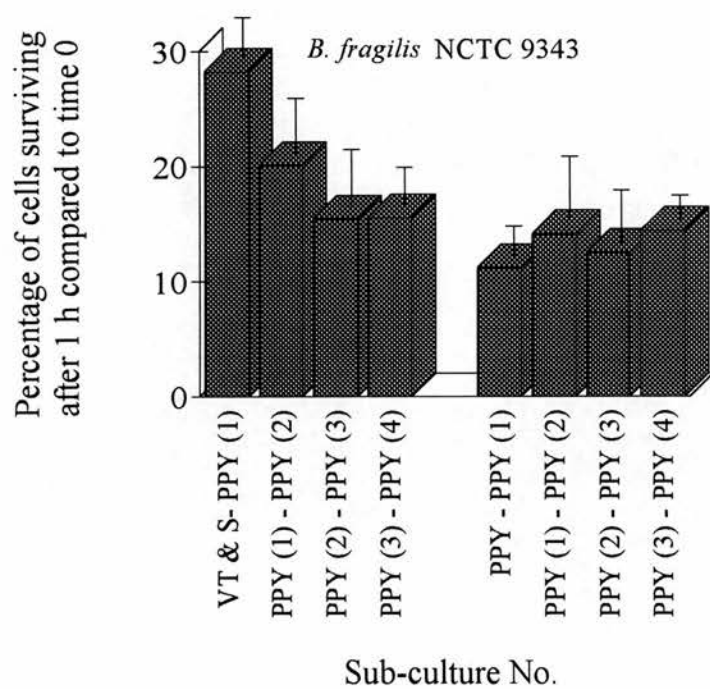
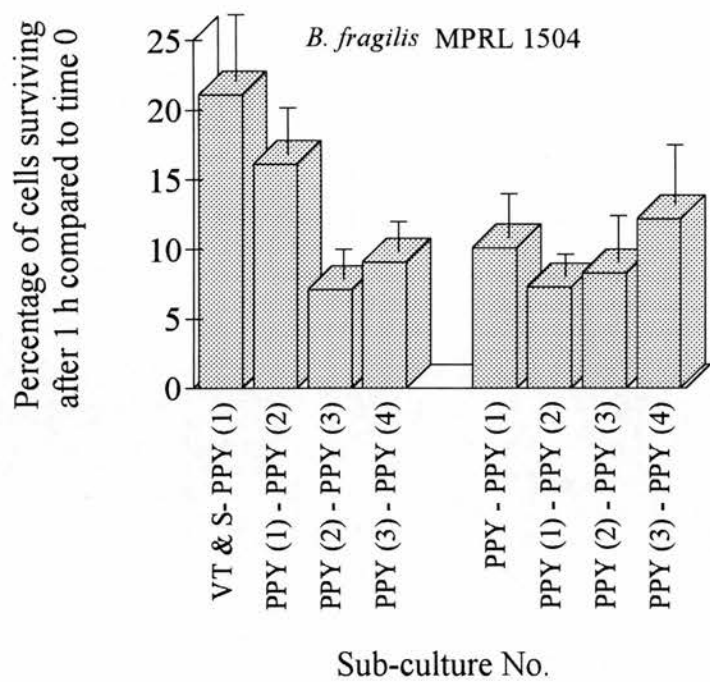


Figure 18. Resistance of *B. fragilis* MPRL 1504 and *B. fragilis* NCTC 9343 to 40% human serum after sub-culture from VT & S or PPY. Results shown are mean percentage survival of bacteria calculated from four replicates. \bar{T} = standard error.

both strains, initial growth in VT & S did appear to increase complement resistance when cells were then sub-cultured into PPY, but this increased resistance was diminished by further sub-cultures of cells into PPY. After two to three sub-cultures into PPY, resistance of cells initially grown in VT & S was no different from those initially grown in PPY.

3.2.3f Complement Resistance of More *Bacteroides fragilis* Strains

It was decided to try to find a *B. fragilis* strain which remained sensitive to complement after growth in VT & S so that its cell surface Ag expression could be compared to that of the strains which became resistant. Twelve further *B. fragilis* strains were therefore tested for their ability to resist complement after growth in PPY and VT & S. These strains had variable resistance to complement after growth in PPY, but all 12 became much more resistant after growth in VT & S (Table 5).

Table 5. Survival of 12 *B. fragilis* strains after 1 h in 40% human serum following growth in PPY and VT & S. Figures shown are mean percentage survival of bacteria calculated from two replicates.

Percent Survival of Bacteria Grown in		
Strain	PPY	VT & S
<i>B. fragilis</i> MPRL 1652	4.4	88.6
<i>B. fragilis</i> MPRL 1978	24.3	95.5
<i>B. fragilis</i> NCTC 9344	35.5	94.2
<i>B. fragilis</i> MPRL 1582	10.1	83.1
<i>B. fragilis</i> MPRL 1583	6.6	88.5
<i>B. fragilis</i> MPRL 1584	14.3	90.2
<i>B. fragilis</i> MPRL 1585	8.1	85.1
<i>B. fragilis</i> MPRL 1586	6.5	94.4
<i>B. fragilis</i> MPRL 1588	10.3	90.2
<i>B. fragilis</i> MPRL 1590	4.8	81.6
<i>B. fragilis</i> MPRL 1981	28.8	90.3
<i>B. fragilis</i> MPRL 1986	30.2	96.7

3.2.4 INVESTIGATION OF THE MECHANISM OF COMPLEMENT RESISTANCE IN *BACTEROIDES THETAIOAOMICRON*

As a *B. fragilis* strain which was sensitive to complement following growth in VT & S could not be found, it was decided to look at the two *B. thetaiotaomicron* strains already tested for complement resistance; one of these strains (MPRL 1959) became resistant to complement after growth in VT & S, whereas the other (NCTC 10582) remained sensitive (Table 4).

Degree of encapsulation, LPS and OMP expression were compared in the two *B. thetaiotaomicron* strains. Differences in degree of encapsulation and in silver-stained LPS profiles between the two strains have already been demonstrated (Figures 8 and 9 respectively). The LPSs were further compared by immunoblotting (Figure 19), and OMP profiles were compared by PAGE followed by Coomassie blue staining and immunoblotting (Figures 20 and 21). Unfortunately, the quality of the LPS immunoblot was poor, possibly due to the age (18 years) of the rabbit serum used. However, some differences were apparent both within each strain grown in different media, and between strains grown in the same media. Interestingly, no bands were present in either strain grown in VT & S. It is not possible to say whether bands visible on the blot were LPS components, or whether they were other material (e.g. protein, other polysaccharides) which may also have been present in aqueous phenol extracts. Differences in OMP profiles were also apparent both by Coomassie blue staining and immunoblotting. There were differences in OMP expression by MPRL 1959 and NCTC 10582 in PPY, and differences in OMP expression in PPY compared to VT & S in NCTC 10582. Immunoblotting gave better resolution of the high *Mr* bands than Coomassie blue. The protein concentration of the extracts from MPRL 1959 grown in VT & S was not strong enough to be visualised by Coomassie blue or immunoblotting.



Figure 19. Immunoblot of polyacrylamide (14%) gel of rapid phenol extracts of *B. thetaiotaomicron* NCTC 10582 and *B. thetaiotaomicron* MPRL 1959 following growth to early stationary phase in PPY, VT and VT & S, using hyperimmune polyclonal rabbit serum raised against *B. thetaiotaomicron* NCTC 10582 whole cells. Tracks: **1**, NCTC 10582 (PPY); **2**, NCTC 10582 (VT); **3**, NCTC 10582 (VT & S); **4**, MPRL 1959 (PPY); **5**, MPRL 1959 (VT); **6**, MPRL 1959 (VT & S).

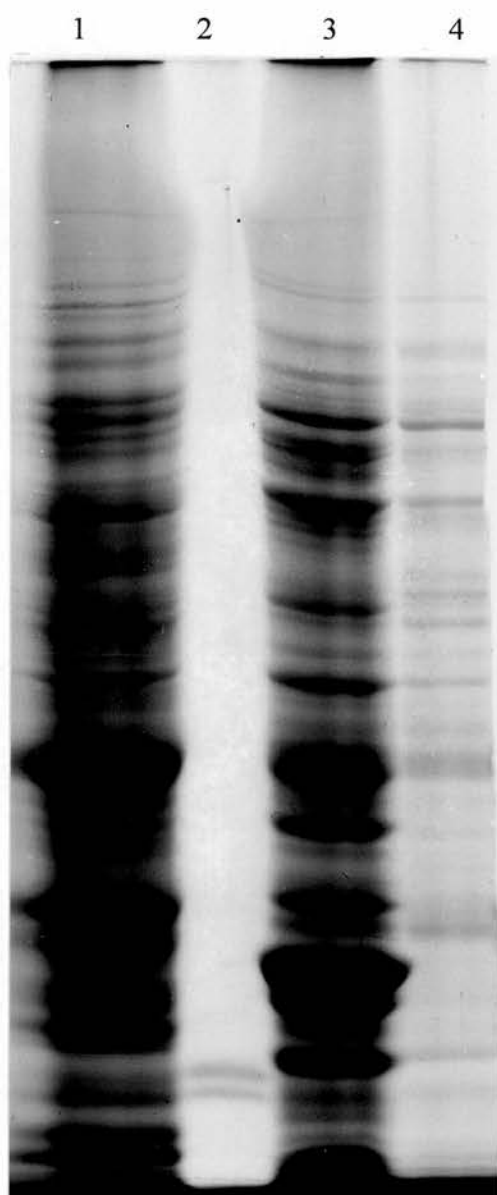


Figure 20. Coomassie blue-stained SDS-PAGE (10% acrylamide) OMP profiles of Sarkosyl extracts of *B. thetaiotaomicron* NCTC 10582 and *B. thetaiotaomicron* MPRL 1959 following growth to early stationary phase in PPY and VT & S. Tracks: **1**, MPRL 1959 (PPY); **2**, MPRL 1959 (VT & S); **3**, NCTC 10582 (PPY); **4**, NCTC 10582 (VT & S). Amounts of protein added per track were: tracks 1 and 3 - 40 μ g; track 2 - 8 μ g; track 4 - 25 μ g.

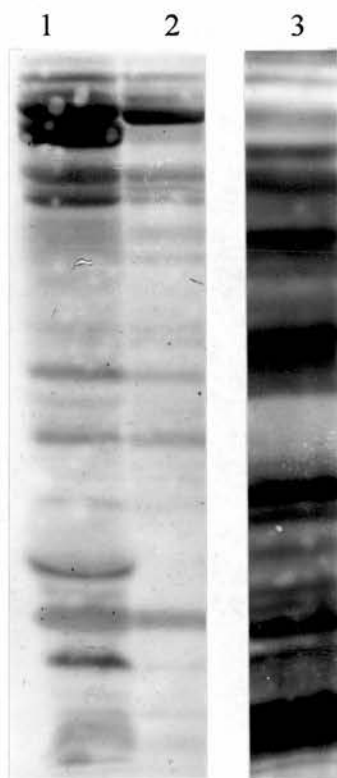


Figure 21. Immunoblot of SDS-polyacrylamide (10%) gel of OMPs from *B. thetaiotaomicron* NCTC 10582 and *B. thetaiotaomicron* MPRL 1959 grown to early stationary phase in PPY and VT & S, using hyperimmune polyclonal rabbit serum raised against *B. thetaiotaomicron* NCTC 10582 OMPs. Tracks: **1**, NCTC 10582 (PPY); **2**, NCTC 10582 (VT & S); **3**, MPRL 1959 (PPY).

3.2.5 CLASSICAL VERSUS ALTERNATIVE PATHWAY ACTIVATION

The aim of this study was to find out whether killing of selected *Bacteroides* strains occurred via the classical or alternative pathway, and to see whether the pathway of complement activation changed in any given strain with growth medium.

3.2.5a Pre-Incubation of Serum with *Bacteroides fragilis* MPRL 1504

Serum was subjected to a series of absorptions with *B. fragilis* MPRL 1504 whole cells grown in PPY or VT as described in Section 2.2.25a in order to absorb out any species-specific Abs. It was then used in a serum sensitivity assay to measure killing of *B. fragilis* MPRL 1504 grown in PPY or VT respectively. Pre-absorption of serum should result in inhibition of the classical pathway, and should indicate whether species-specific Abs in serum are necessary to activate the complement cascade. The results are shown in Figure 22. There was a reduction in bacterial killing following pre-incubation of serum with bacteria, with cells grown in PPY and VT. The reduction in killing was greater in PPY-grown cells, suggesting that killing of these cells was more dependent on the classical complement pathway, and on specific Ab, than killing of VT-grown cells.

A potential problem with this experiment was that there could have been anti-bacteroides Abs present in the HISS, which would stick to the cells during growth. If they were not removed during the washing process, they would opsonise the bacteria, and could give false positive results - i.e. they could activate the complement cascade rather than Abs present in human serum. Therefore whole cells of *B. fragilis* MPRL 1504 grown in VT & S were coated onto ELISA plates, and IgG levels in HISS were measured. There was anti-*B. fragilis* IgG present in the sheep serum, comparable to levels seen in low titre human serum (see Section 3.4). Therefore the possibility that the sheep Abs were opsonising the bacteria could not be ruled out.

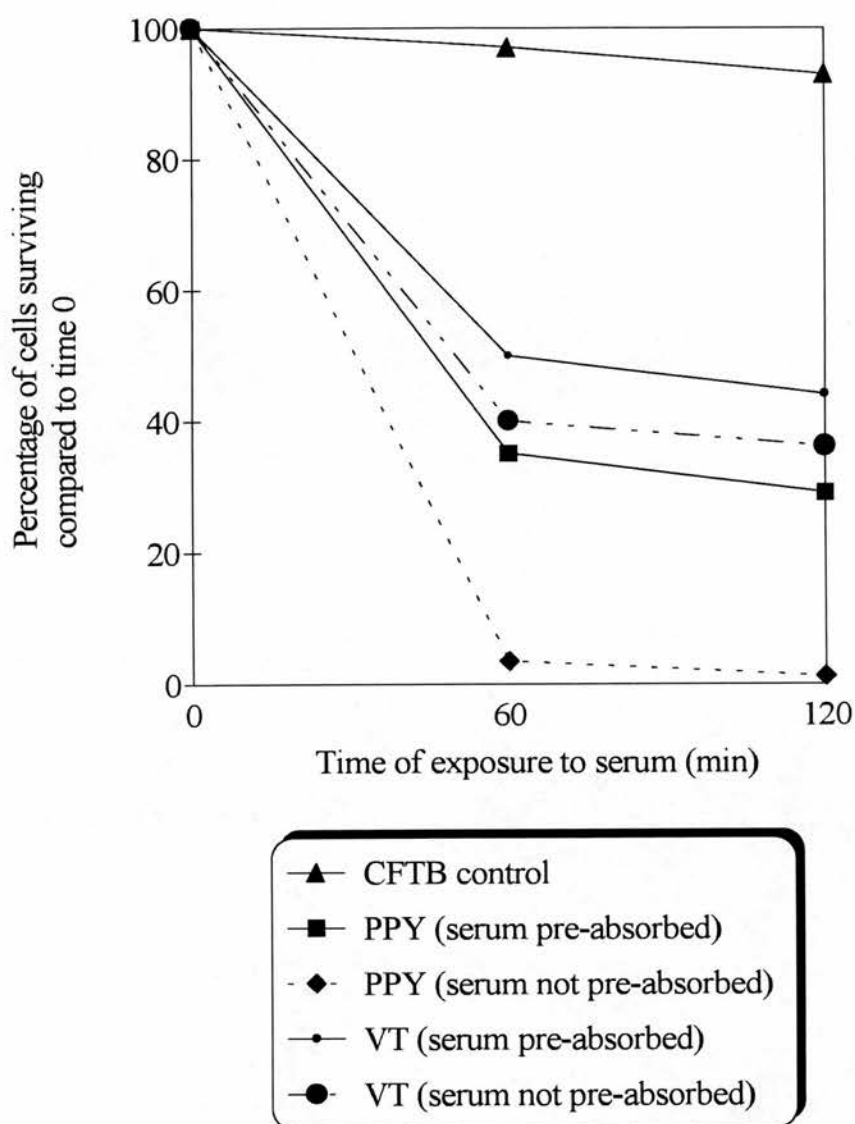


Figure 22. Killing of *B. fragilis* MPRL 1504 after growth in PPY and VT by 40% human serum. Serum was subjected to a series of absorptions with *B. fragilis* MPRL 1504 whole cells grown in PPY or VT prior to using it in the complement sensitivity assay in order to remove specific Ab.

3.2.5b Treatment of Serum with EGTA

To try to overcome the drawbacks of the above experiment, EGTA was added to serum prior to using it in the complement sensitivity assay, in order to chelate Ca^{2+} ions and therefore specifically inactivate the classical pathway. Several *Bacteroides* strains were tested, some of which became resistant to complement after growth in VT & S, and others of which remained sensitive (Figure 23). Addition of EGTA to serum reduced bacterial killing in all cases, indicating that the classical pathway was being activated. However, the degree of activation of the classical and alternative pathways both between strains grown in the same media, and within a given strain grown in different media, varied considerably. In all strains, the biggest reduction in killing with EGTA was in cells grown in PPY, followed by cells grown in VT, followed by cells grown in VT & S. This indicated that classical pathway activation was greatest for PPY-grown cells, followed by VT-grown cells, followed by VT & S-grown cells. The opposite was true for alternative pathway activation. Addition of EGTA to serum practically abolished killing of *B. variabilis* VPI 11368 grown in PPY, indicating that complement activation in this case was more or less exclusively via the classical pathway. In the other strains grown in PPY, killing was reduced in the presence of EGTA, but not completely abolished, indicating that complement activation was occurring partly via the classical, and partly via the alternative pathway. *B. vulgatus* MPRL 1985 showed the smallest reductions in killing in the presence of EGTA, suggesting that complement activation in this case was mainly via the alternative pathway. There was no obvious correlation between pathway of activation and degree of resistance to complement.

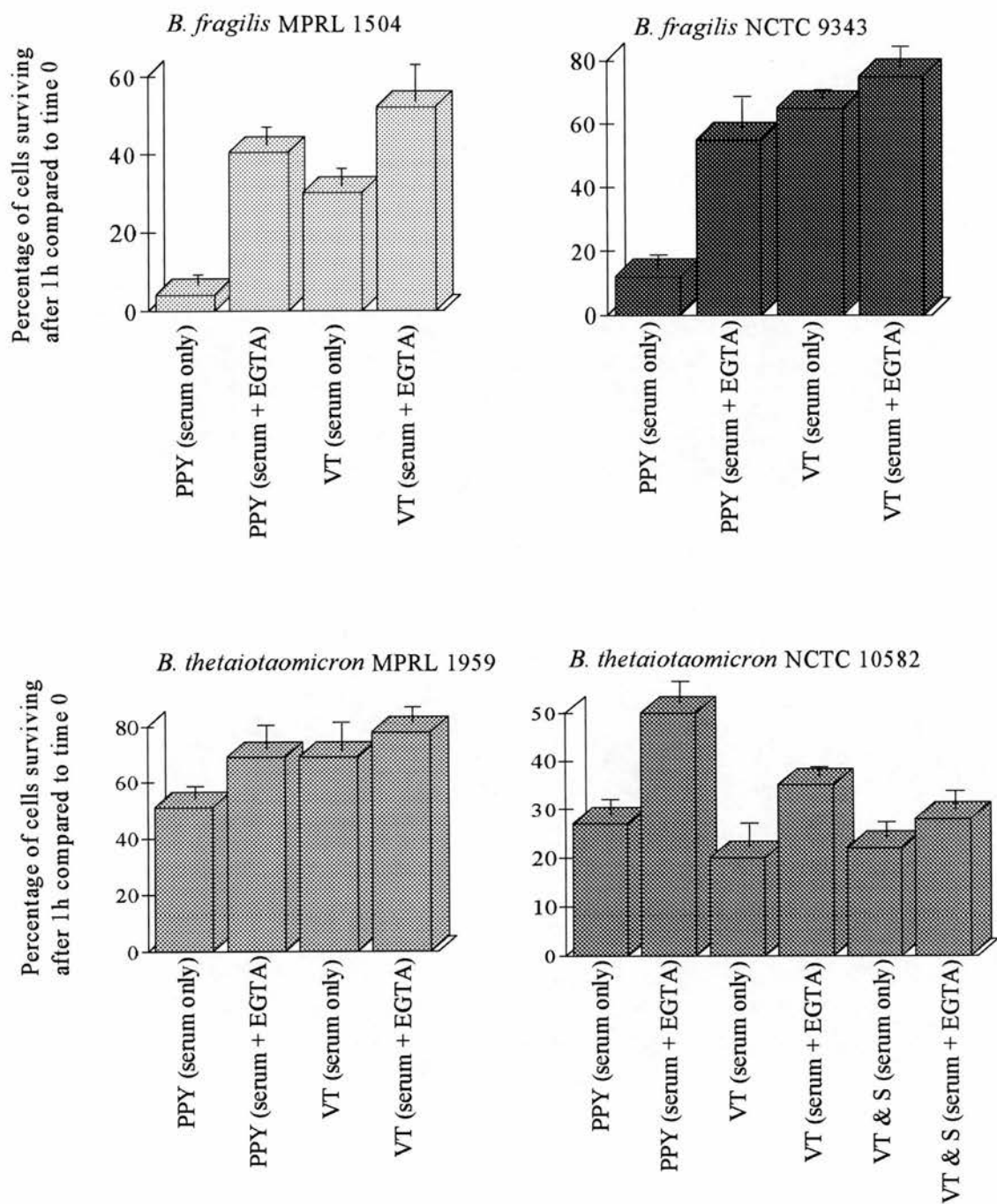


Figure 23. Survival of selected *Bacteroides* strains following growth in PPY, VT and VT & S in 40% human serum and in 40% human serum + EGTA. Results shown are average percentages from at least two replicates. \bar{T} = standard error.

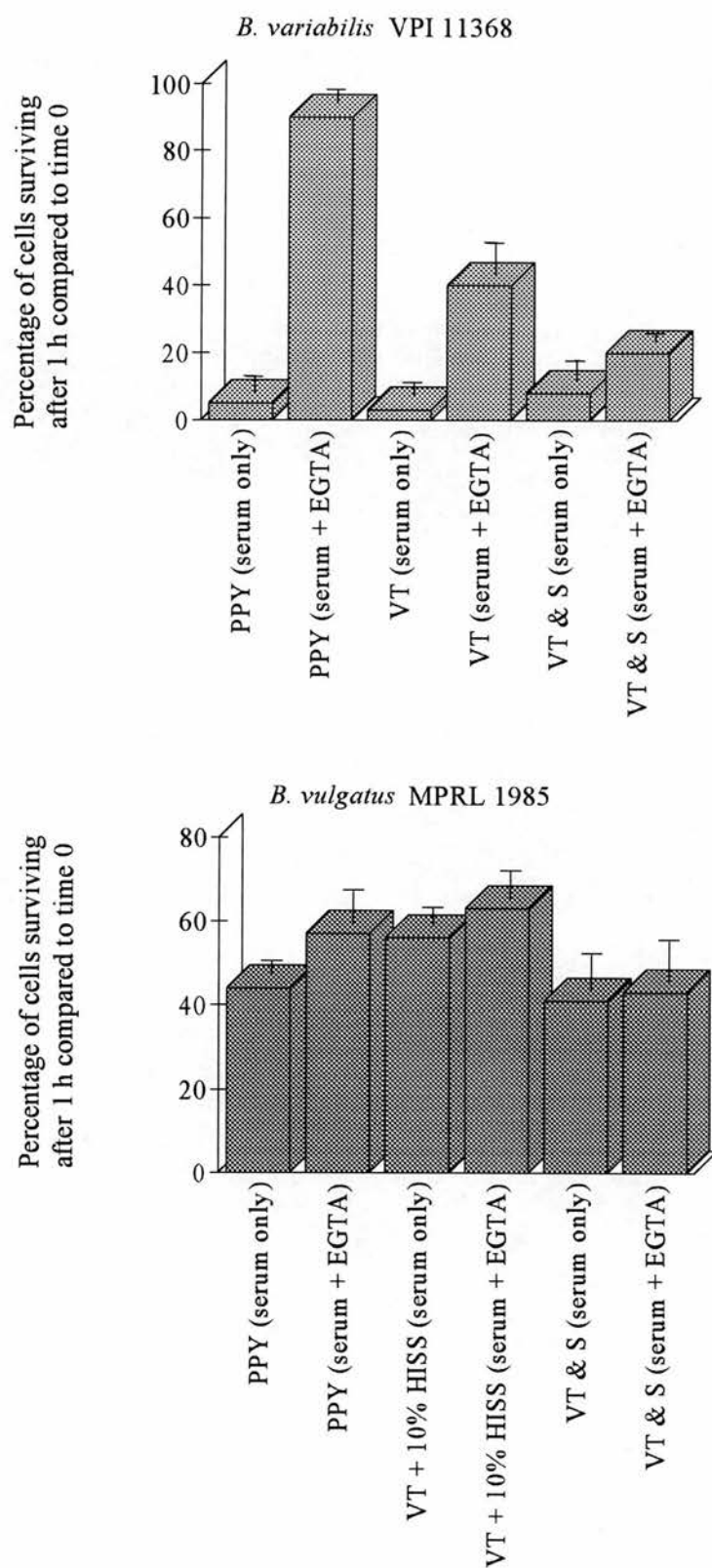


Figure 23 (continued).

3.2.6 BACTERIAL KILLING BY SERUM FROM SYSTEMIC INFLAMMATORY RESPONSE SYNDROME PATIENTS

Whole blood was obtained from two patients with SIRS and the serum processed by method A. Each serum sample was then tested for its ability to kill *B. fragilis* MPRL 1504 grown in PPY, and the haemolytic complement values (CH50) were measured. Bactericidal capabilities and CH50 values were compared to those of four healthy volunteers (Table 6). Bacterial killing and CH50 values were considerably reduced in both patients' sera compared to serum from healthy individuals. Although there was considerable variation in CH50 values between the healthy individuals' sera, all of these sera killed the bacteria with approximately the same efficiency, probably because they were all within the normal (50-150 units / ml) CH50 range. On the other hand, the CH50 values (and therefore complement levels) of the SIRS patients' sera were well below the normal range, the result of which was less efficient bacterial killing.

Table 6. Survival of *B. fragilis* MPRL 1504 grown in PPY in 10% and 40% serum from SIRS patients and healthy individuals. Results shown are means calculated from two replicates.

Percentage survival of <i>B. fragilis</i> MPRL 1504 after 1 h in:			
Serum from:	CH50 (units / ml)	10% serum	40% serum
Volunteer 1	56.2	8.9	7.8
Volunteer 2	123.0	13.4	10.2
Volunteer 3	83.2	12.0	9.0
Volunteer 4	59.1	12.2	8.3
SIRS patient 1	23.9	31.3	18.4
SIRS patient 2	31.6	43.0	20.2

3.3 ANALYSIS OF THE SURFACE POLYSACCHARIDE COMPLEX OF BACTEROIDES FRAGILIS

There is much controversy and confusion surrounding the composition and structure of the surface polysaccharide complex of *B. fragilis*. This confusion has led to many different interpretations of results, particularly with regard to possible roles of surface polysaccharides in pathogenicity. In addition, it was suspected in earlier experiments performed in this thesis that the aqueous phenol extracts were not pure LPS. The aim of these experiments was to try to determine what was present in the surface polysaccharide complex of *B. fragilis*, and to try to purify and analyse the different components, in an attempt to address some of the unresolved questions which surround this subject.

3.3.1 COMPARISON OF ULTRACENTRIFUGED AND NON-ULTRACENTRIFUGED AQUEOUS PHENOL EXTRACTS

Aqueous phenol extracts of *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 grown in PPY, VT and VT & S were examined in a variety of ways. Initially, each extract was divided into two; one half was subjected to the final rotary evaporation and ultracentrifugation steps in the extraction method (Hancock & Poxton 1988), whilst the other half was lyophilised without these final steps, and percentage yields were calculated. In case material was present in the phenol phase, this was also collected and lyophilised without ultracentrifugation. The ultracentrifuged and non-ultracentrifuged samples were then assayed for protein, carbohydrate and phosphorous, and were compared by PAGE followed by silver staining and immunoblotting.

3.3.1a Percentage Yields of Extracts

Percentage yields of the ultracentrifuged and non-ultracentrifuged aqueous phenol extracts from cells grown in the three media were calculated (Table 7). In all cases,

the yield of material from the phenol phase was very low. For both strains, the lowest yield in the aqueous phase was from cells grown in PPY, then in VT & S, then in VT, for both ultracentrifuged and non-ultracentrifuged extracts. In all cases except MPRL 1504 grown in VT & S, the percentage yield from the non-ultracentrifuged extracts was greater than the yield from the ultracentrifuged ones.

Table 7. Percentage yields of ultracentrifuged and non-ultracentrifuged aqueous phenol extracts of *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 grown in PPY, VT and VT & S.

Strain & growth medium	Phenol phase (not ultracentrifuged)	Aqueous phase (not ultracentrifuged)	Aqueous phase (ultracentrifuged)
	% yield*	% yield*	% yield*
NCTC 9343 (PPY)	0.01	0.59	0.56
NCTC 9343 (VT)	0.01	1.33	0.75
NCTC 9343 (VT & S)	0.02	0.89	0.63
MPRL 1504 (PPY)	0.02	0.7	0.63
MPRL 1504 (VT)	0.03	2.02	1.15
MPRL 1504 (VT & S)	0.02	0.86	0.88

* (weight of lyophilised aqueous phenol extract ÷ weight of lyophilised cells) x 100.

3.3.1b Polyacrylamide Gel Electrophoresis of Extracts

Ultracentrifuged and non-ultracentrifuged aqueous phase extracts, and phenol phase extracts were compared by PAGE followed by silver staining (Figure 24) and immunoblotting (Figure 25). There was only sufficient material from the phenol phases to run on one gel, and this was stained with silver; the resultant profiles were similar to the profiles from the aqueous layers, except weaker (not shown). Silver

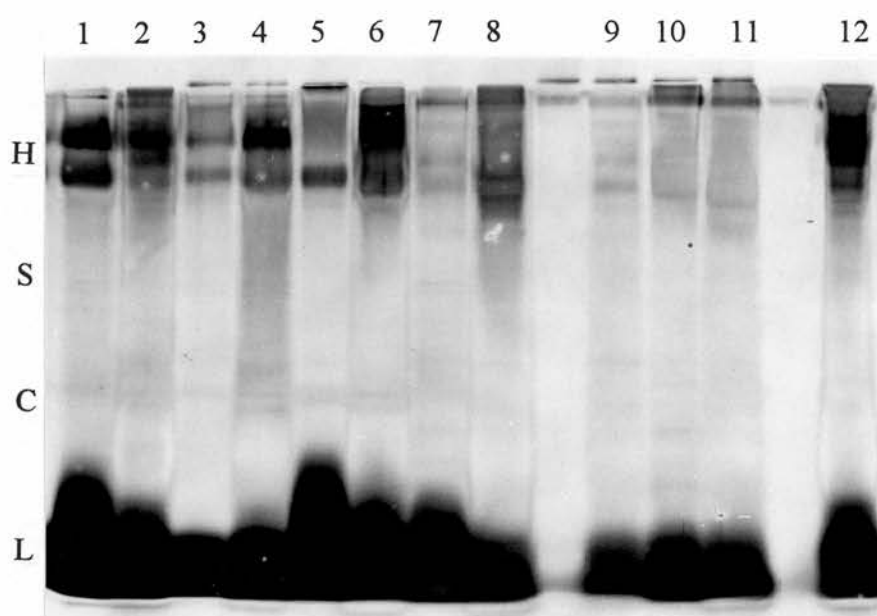


Figure 24. Silver-stained PAGE (14%) profiles of ultracentrifuged (+ uc) and non-ultracentrifuged (- uc) aqueous phenol extracts (50 μ g / track) of *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 grown to early stationary phase in PPY, VT and VT & S. Tracks: **1**, NCTC 9343 (+ uc, PPY); **2**, NCTC 9343 (- uc, PPY); **3**, NCTC 9343 (+ uc, VT); **4**, NCTC 9343 (- uc, VT); **5**, NCTC 9343 (+ uc, VT & S); **6**, NCTC 9343 (- uc, VT & S); **7**, MPRL 1504 (+ uc, PPY); **8**, MPRL 1504 (- uc, PPY); **9**, MPRL 1504 (+ uc, VT); **10**, MPRL 1504 (- uc, VT); **11**, MPRL 1504 (- uc, VT & S); **12**, MPRL 1504 (+ uc, VT & S). Abbreviations as in Figure 9a.

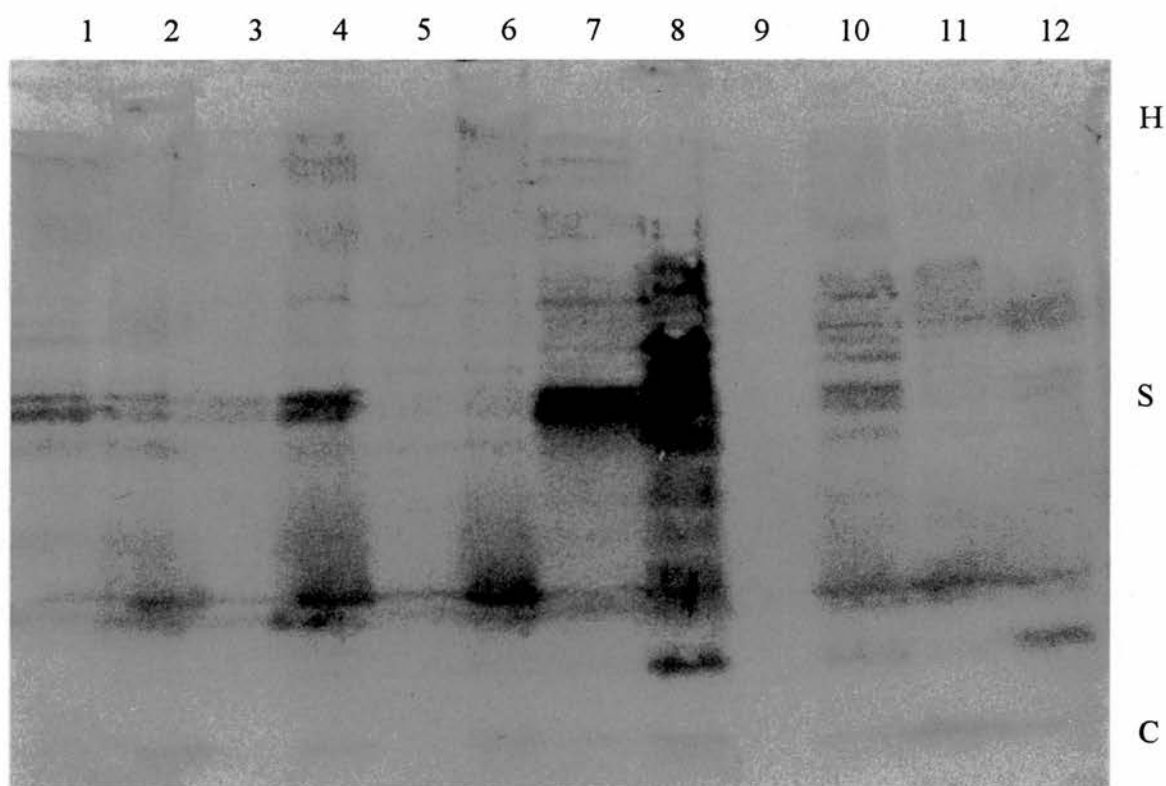


Figure 25. Immunoblot of polyacrylamide (14%) gel of ultracentrifuged (+ uc) and non-ultracentrifuged (- uc) aqueous phenol extracts (100 μ g / track) of *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 grown to early stationary phase in PPY, VT and VT & S, using hyperimmune rabbit serum raised against *B. fragilis* MPRL 1504 whole cells. Tracks: 1, NCTC 9343 (+ uc, PPY); 2, NCTC 9343 (- uc, PPY); 3, NCTC 9343 (+ uc, VT); 4, NCTC 9343 (- uc, VT); 5, NCTC 9343 (+ uc, VT & S); 6, NCTC 9343 (- uc, VT & S); 7, MPRL 1504 (+ uc, PPY); 8, MPRL 1504 (- uc, PPY); 9, MPRL 1504 (+ uc, VT); 10, MPRL 1504 (- uc, VT); 11, MPRL 1504 (+ uc, VT & S); 12, MPRL 1504 (- uc, VT & S). Abbreviations as in Figure 9a.

staining of the aqueous phase ultracentrifuged and non-ultracentrifuged extracts revealed some differences. In both strains grown in PPY and VT & S, ultracentrifuged extracts had a greater amount of low *Mr* material present than non-ultracentrifuged extracts, whereas in both strains grown in VT, the amount of low *Mr* material in ultracentrifuged and non-ultracentrifuged extracts was approximately equal. There were differences in all cases between the high *Mr* material in the ultracentrifuged, compared to the non-ultracentrifuged extracts. In some cases (NCTC 9343 in VT & S, MPRL 1504 in PPY and MPRL 1504 in VT & S) the amount of high *Mr* material was greater in non-ultracentrifuged extracts. Generally, the high *Mr* material was less well defined in non-ultracentrifuged extracts. The non-ultracentrifuged extracts of the two strains grown in VT had a greater amount of poorly defined, dark staining material in the smooth LPS region compared to the ultracentrifuged extracts. No obvious ladder patterns were evident in any of the extracts.

The quality of staining of the immunoblot was poor. In all cases except NCTC 9343 grown in PPY, the non-ultracentrifuged extracts stained stronger than the ultracentrifuged extracts. Material from the ultracentrifuged extract of MPRL 1504 in VT did not stain at all. Material below the common Ag did not stain in any extract. Evenly spaced bands, which could have possibly been a ladder pattern, were evident in the ultracentrifuged extract of MPRL 1504 grown in VT & S. It is possible that there were ladder patterns present in some of the other extracts which were not visible due to the poor quality of the blot.

3.3.1c Estimation of Protein, Carbohydrate and Organic Phosphorus in Extracts

The relative amounts of protein, carbohydrate (neutral sugars) and organic phosphorus were measured in the ultracentrifuged and non-ultracentrifuged aqueous phenol extracts of *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 grown in the

three media. The results are in Figure 26a-b. To standardise all results, concentrations were worked out as a percentage of the dry weight of the extract. In the non-ultracentrifuged extracts compared to the ultracentrifuged ones, there was a relatively greater amount of protein and a smaller amount of carbohydrate. In two cases (NCTC 9343 in VT & S and MPRL 1504 in PPY) phosphorus levels were noticeably greater in non-ultracentrifuged extracts compared to ultracentrifuged ones, whilst in the other cases phosphorus levels were very similar in non-ultracentrifuged and ultracentrifuged extracts. These results suggest that ultracentrifugation did not pellet out all of the protein in the extracts. This experiment also confirmed earlier suspicions that the aqueous phenol extracts were not pure LPS, but also contained protein.

As a control, the non-ultracentrifuged extract of *B. fragilis* NCTC 9343 grown in PPY was assayed for KDO without prior dephosphorylation. The amount present was extremely low (0.03%).

It was decided in all future experiments to use non-ultracentrifuged aqueous phenol extracts, as ultracentrifugation did not purify the LPS and it was possible that important antigenic determinants were being lost as a result of the ultracentrifugation process.

3.3.2 TREATMENT OF AQUEOUS PHENOL EXTRACTS WITH PROTEINASE K AND PERIODATE

The non-ultracentrifuged aqueous phenol extracts were further examined by PAGE, silver staining and immunoblotting following digestion with Proteinase K (to destroy protein) and periodate (to destroy carbohydrate). The silver-stained gel is shown in Figure 27. Proteinase K treatment did not make a large difference to the aqueous phenol extract profiles, but in two cases (NCTC 9343 in VT & S and MPRL 1504 in

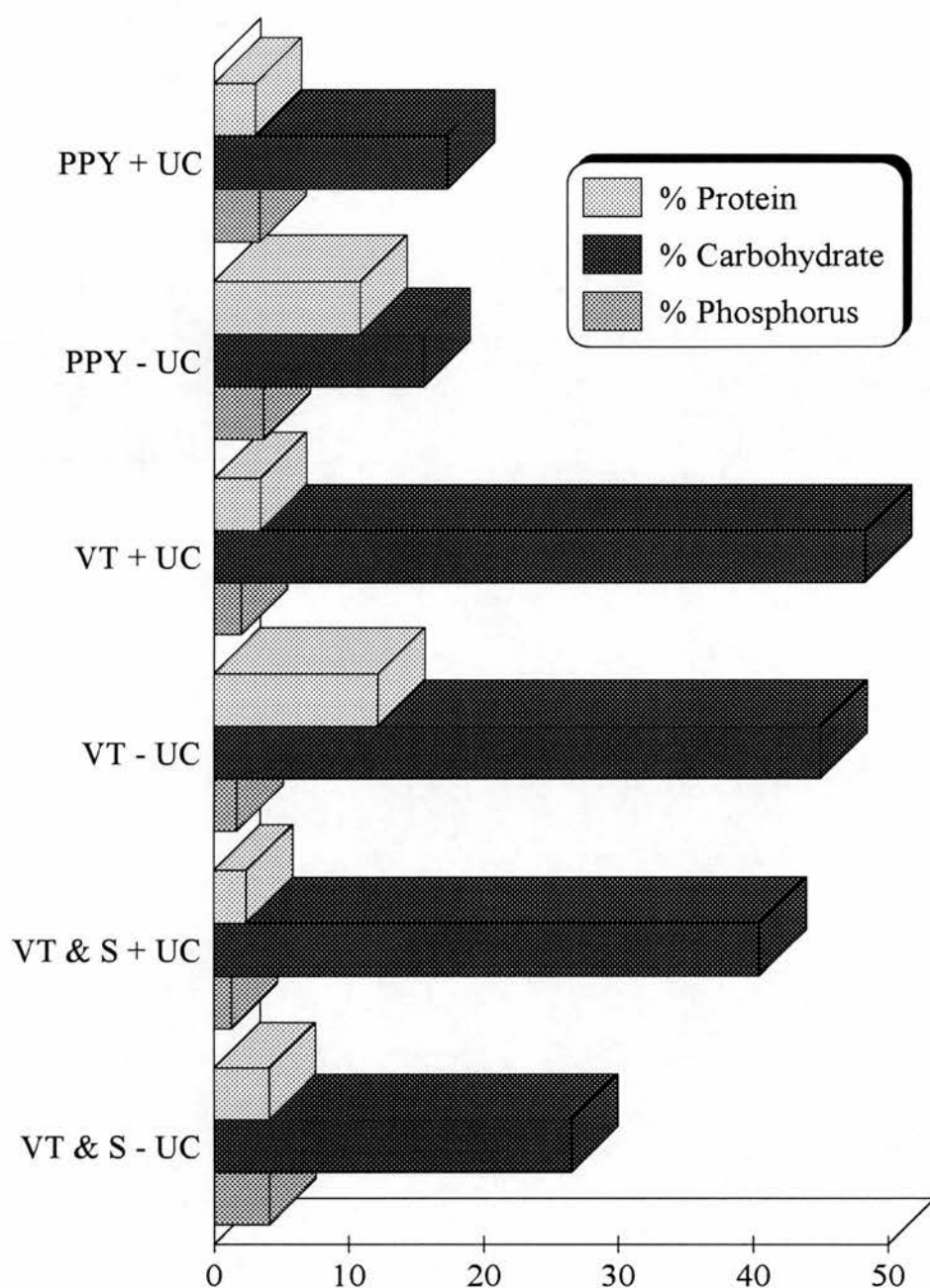


Figure 26a. Percentage of protein, carbohydrate and organic phosphorus in ultracentrifuged (+ UC) and non-ultracentrifuged (- UC) aqueous phenol extracts of *B. fragilis* NCTC 9343 grown to early stationary phase in three media. Results shown are means calculated from at least two replicates.

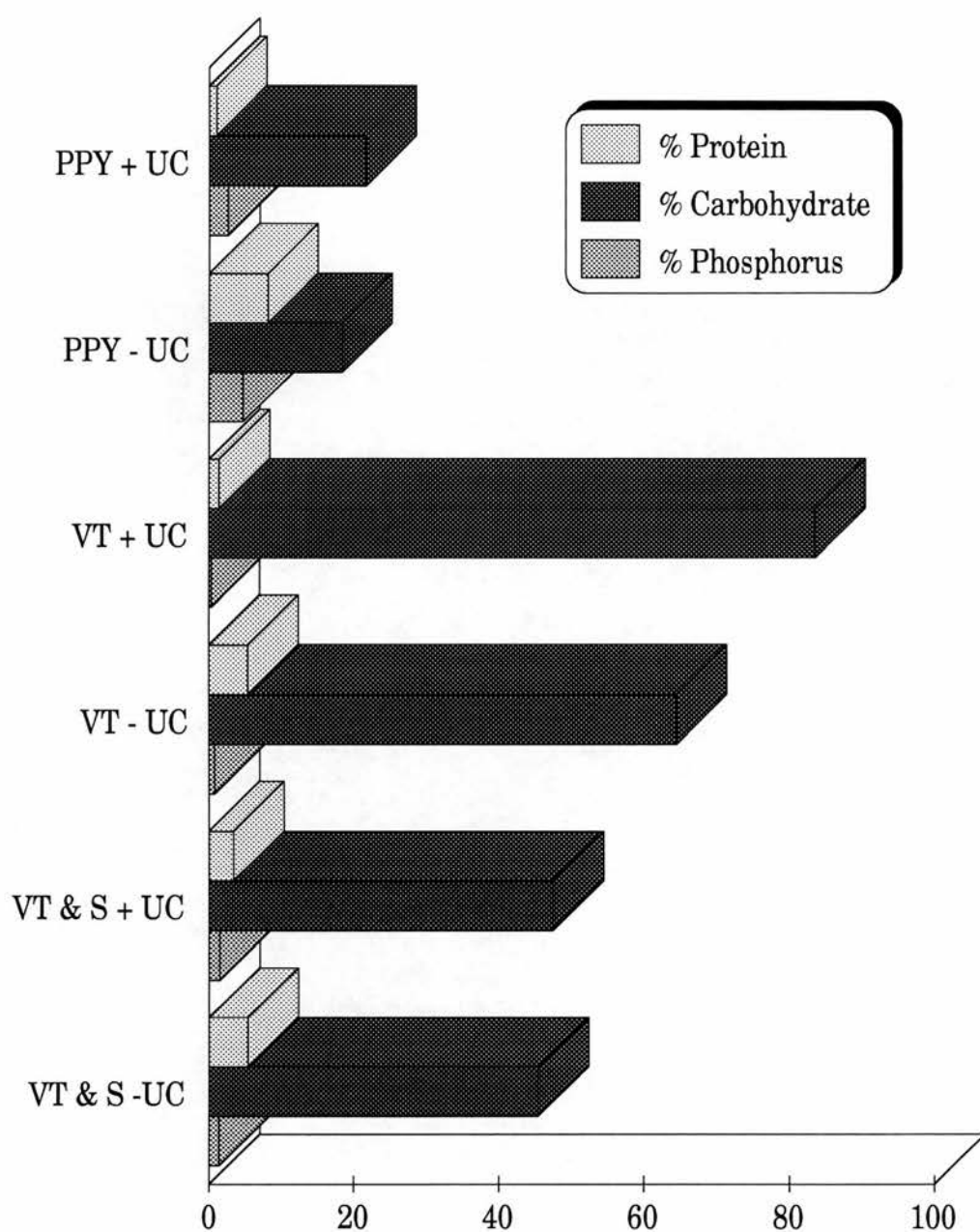


Figure 26b. Percentage of protein, carbohydrate and organic phosphorus in ultracentrifuged and non-ultracentrifuged aqueous phenol extracts of *B. fragilis* MPRL 1504 grown to early stationary phase in three media. Results shown are means calculated from at least two replicates.

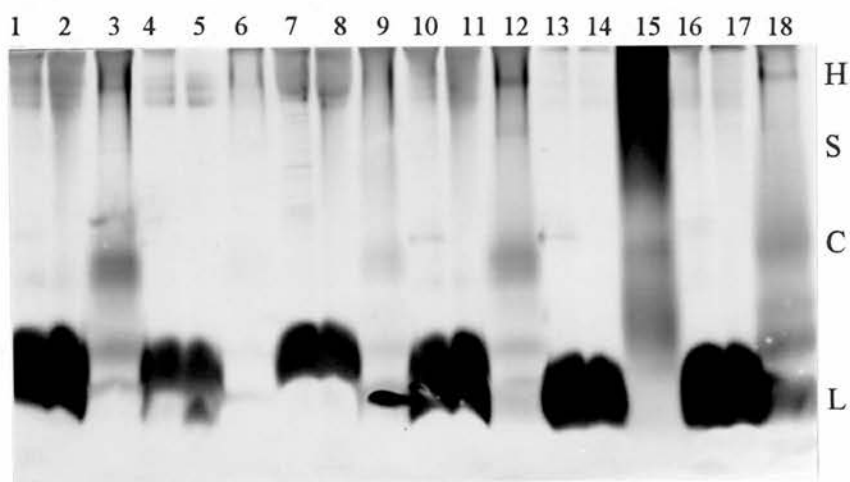


Figure 27. Silver-stained PAGE (14% acrylamide) LPS profiles of aqueous phenol extracts (100 μg / track) of *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 grown to early stationary phase in PPY, VT and VT & S. Extracts were treated with Proteinase K (PrK), periodate (Per) or untreated (Utd). Tracks: **1**, NCTC 9343 (PPY, Utd); **2**, NCTC 9343 (PPY, PrK); **3**, NCTC 9343 (PPY, Per); **4**, NCTC 9343 (VT, Utd); **5**, NCTC 9343 (VT, PrK); **6**, NCTC 9343 (VT, Per); **7**, NCTC 9343 (VT & S, Utd); **8**, NCTC 9343 (VT & S, PrK); **9**, NCTC 9343 (VT & S, Per); **10**, MPRL 1504 (PPY, Utd); **11**, MPRL 1504 (PPY, PrK); **12**, MPRL 1504 (PPY, Per); **13**, MPRL 1504 (VT, Utd); **14**, MPRL 1504 (VT, PrK); **15**, MPRL 1504 (VT, Per); **16**, MPRL 1504 (VT & S, Utd); **17**, MPRL 1504 (VT & S, PrK); **18**, MPRL 1504 (VT & S, Per). Abbreviations as in Figure 9a.

PPY) it removed unevenly-spaced bands in the smooth LPS region. It did not remove the very high, or low *Mr* material. The common Ag was not visible on this gel in all cases, but in those cases where it was visible (NCTC 9343 in PPY and MPRL 1504 in all media) it disappeared following Proteinase K treatment. Periodate destroyed the low *Mr* material either completely or almost completely in all cases. It also either destroyed, or considerably reduced the amount of the high *Mr* material. The common Ag band had disappeared following periodate treatment, but immediately below the region where the common Ag was in the untreated samples, there was a larger, more diffuse band present in the periodate-treated samples. Immunoblotting using hyperimmune rabbit serum raised against *B. fragilis* MPRL 1504 whole cells failed to show any material in the Proteinase K and periodate-treated samples (gel not shown). Material in the untreated lanes was as previously seen in Figure 25.

An attempt was made to estimate the amount of carbohydrate remaining in the aqueous phenol extracts following periodate treatment. However, this was unsuccessful as the periodate present in the samples caused the final colour in the carbohydrate assay to be brown rather than yellow. This brown colour had a different absorption maximum to the usual yellow end colour, and so the sample could not be adequately compared with the control. It was not possible to carry out a protein assay on the Proteinase K-treated samples, as the presence of Proteinase K would have given a positive result.

3.3.3 FRACTIONATION OF THE SURFACE POLYSACCHARIDE COMPLEX OF *BACTEROIDES FRAGILIS* NCTC 9343

An attempt was made to separate and purify the different components of the surface polysaccharide complex of *B. fragilis* NCTC 9343, so that the different components could then be analysed. An aqueous phenol extract of *B. fragilis* NCTC 9343 was partially purified and fractionated into different components as described in Section

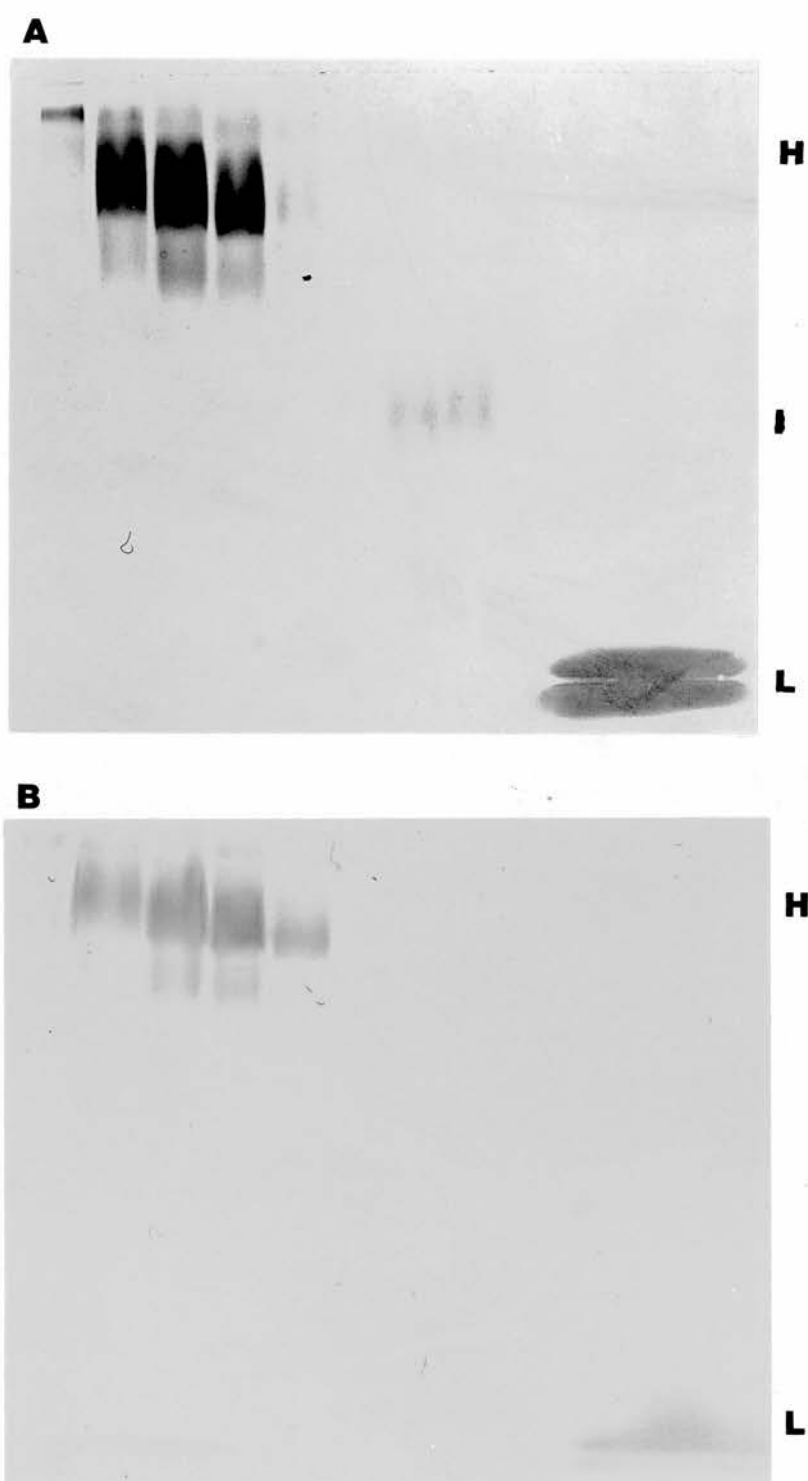


Figure 28. Silver-stained PAGE (10% acrylamide) profiles of fractions of a partially purified aqueous phenol extract of *B. fragilis* NCTC 9343. Fractions were separated by gel filtration chromatography. Abbreviations: H = high *Mr* material, I = intermediate *Mr* material, L = low *Mr* material.

2.2.9. The first fractionation was carried out on a gel filtration (Sephacryl S-300) column; fractions were collected and visualised by PAGE and silver staining. The material to be separated was passed through the column in two batches, and the resultant gels are shown in Figure 28a-b. Three different classes of material were evident; high *Mr*, intermediate *Mr* and low *Mr*. There is no intermediate *Mr* material visible in Figure 28b; it was visible on the gel, but not picked up on the photograph. The gel represented in Figure 28b had a faint line at the dye front in the same tracks as the high *Mr* material.

Half of the high *Mr* material (assumed to be CP) was subjected to acid hydrolysis to separate it into its two polysaccharide components - PSA and PSB. These were then separated on an ion exchange column (DEAE-Sephacel).

3.3.4 ESTIMATION OF PROTEIN, CARBOHYDRATE AND ORGANIC PHOSPHORUS IN SURFACE POLYSACCHARIDE FRACTIONS

The relative amounts of protein, carbohydrate and organic phosphorus were measured in the surface polysaccharide fractions. The results are in Figure 29. To standardise all results, the concentrations were worked out as a percentage of the dry weight of the extract. The amount of carbohydrate was highest in the high *Mr* fraction and lowest in the intermediate *Mr* fraction. Amounts of protein were very low in all fractions except the intermediate *Mr* fraction. Organic phosphorus levels were very low in all fractions except the low *Mr* fraction. The phosphorus level in PSA and PSB was much lower than that in the low *Mr* fraction; it is possible that acid hydrolysis of the low *Mr* material, and the subsequent ion exchange chromatography were responsible for this reduction.

The low *Mr* fraction was assayed for KDO content without prior dephosphorylation. The amount was extremely low (0.02%).

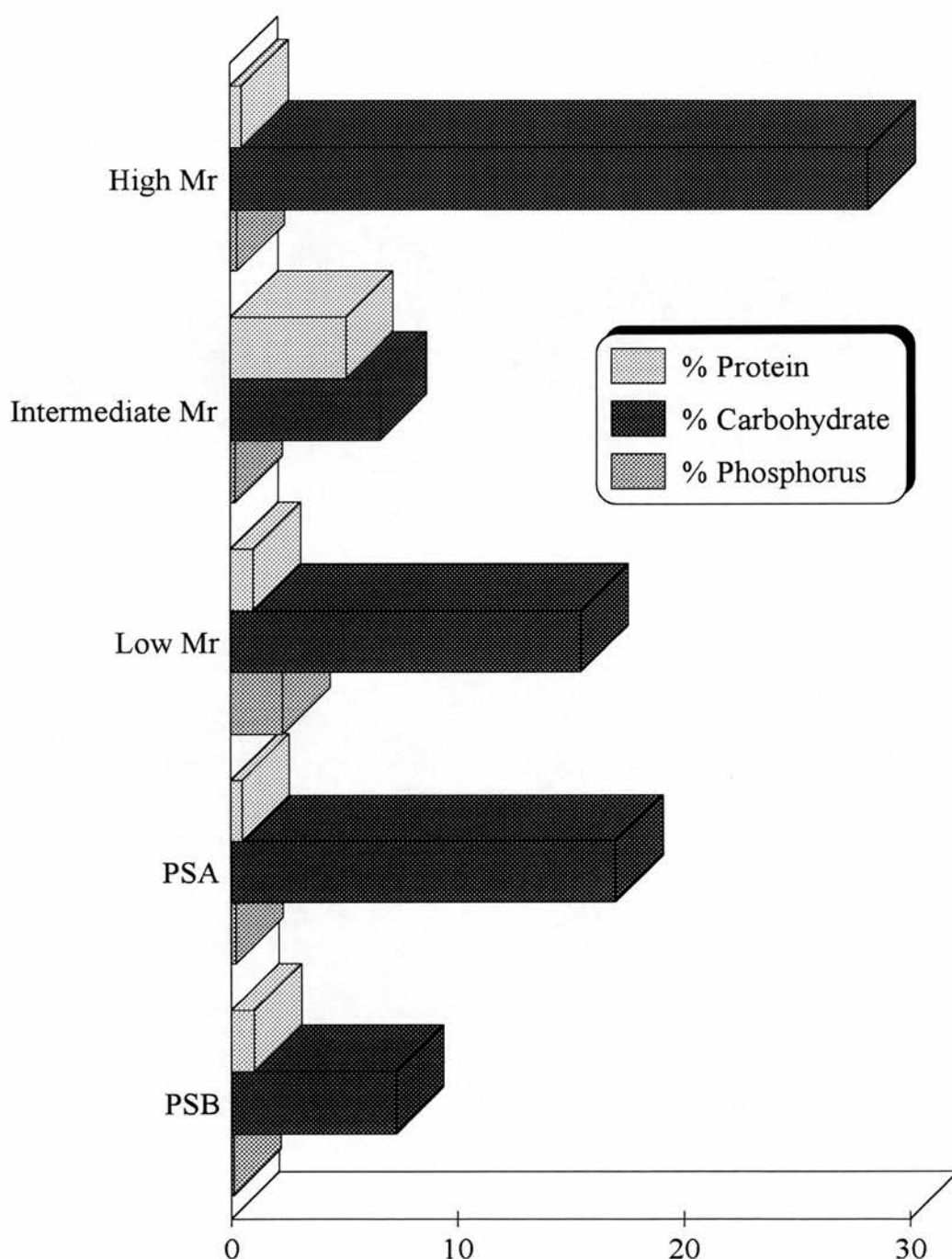


Figure 29. Percentage of protein, carbohydrate and organic phosphorus in surface polysaccharide fractions of *B. fragilis* NCTC 9343. Results are means calculated from at least two replicates.

3.3.5 CHARACTERISATION OF AN AQUEOUS PHENOL EXTRACT AND SURFACE POLYSACCHARIDE FRACTIONS OF *BACTEROIDES FRAGILIS* NCTC 9343 BY DOT BLOTTING AND IMMUNOBLOTTING

To try to characterise the surface polysaccharide complex of *B. fragilis* NCTC 9343 further, a non-ultracentrifuged aqueous phenol extract of the bacterium grown in PPY, and the surface polysaccharide fractions were probed with hyperimmune rabbit serum and with a panel of MAbs which reacted specifically against the surface Ags of *B. fragilis* NCTC 9343 (listed in Table 2). Dot blotting, and subsequently immunoblotting following PAGE were used.

3.3.5a Dot Blotting

The results of the dot blotting are shown in Table 8. The reactivities of the Abs with the aqueous phenol extract were very similar to the reactivities with the high *Mr* fraction. All of the Abs except 3F6 (against an undefined epitope in the SC population) reacted with the aqueous phenol extract, and all of the Abs except 6G3 (possibly against the common Ag) and 3F6 reacted with the high *Mr* fraction. The only Ab to react with all fractions was the rabbit serum, and PSA and the low *Mr* fraction only reacted with the rabbit serum. Of the characterised MAbs, PSB and the intermediate *Mr* fraction reacted with those against the LC and EDL (3D7 and 4C5), and in addition the intermediate *Mr* fraction reacted with 3C8 (against the O-Ag). Fractionation of the high *Mr* material into PSA and PSB considerably reduced their reactivity with the MAbs.

Dot blotting was useful to show patterns of reactivity of the different Abs with the cell surface fractions, but could not give any more information. Therefore to attempt to characterise the cell surface Ags further, it was decided to examine them by PAGE and immunoblotting.

Table 8. Reactivities of polyclonal rabbit serum and MAbs against an aqueous phenol extract and different surface polysaccharide fractions of *B. fragilis* NCTC 9343 by dot blotting.

	<u>Antibody</u>													
	R	3C8	3H5	3D7	6G3	5B7	2B8	1A4	3C10	4C5	3F6 #	3F6 *	1E2	2E12
AP extract	+++	+++	+++	+++	+/-	++	+++	+++	+++	+++	-	-	+++	+
High <i>Mr</i>	++	+++	+++	++	-	++	+++	+++	+++	+++	-	-	++	+
PSA	++	-	-	-	-	-	-	-	-	-	-	-	-	-
PSB	++	-	-	+/-	-	+/-	+	-	+/-	+/-	-	-	-	-
Intermed. <i>Mr</i>	+	+	+/-	+	-	-	+/-	+	+	+	-	-	+	-
Low <i>Mr</i>	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Abbreviations: R = Rabbit polyclonal hyperimmune serum against *B. fragilis* MPRL 1504 whole cells, # = 2 day supernate, * = 7 day supernate, AP = aqueous phenol, +++ = strong reaction to - = no reaction.

3.3.5b Immunoblotting

The aqueous phenol extract and the surface polysaccharide fractions were electrophoresed and were then probed with the Abs which gave a positive reaction in the dot blots. In addition, they were probed with polyclonal rabbit sera raised against *B. fragilis* NCTC 9343, *B. fragilis* NCTC 9344 and the *B. fragilis* common Ag.

Initially the samples were electrophoresed and stained with silver. This was done in order to find the percentage of acrylamide in the separating gel, and the distance to let the samples run in the separating gel which gave good separation of the different fractions, but which did not separate them so much that they became very diffuse and difficult to see. It was found that running the samples approximately 5 cm down an

8% acrylamide separating gel gave the best resolution (Figure 30). PSB did not stain with silver.

Multiblots of the aqueous phenol extract, and the high *Mr* material are shown in Figure 31a-b. Immunoblotting of the aqueous phenol extract with polyclonal rabbit sera against NCTC 9344 whole cells, NCTC 9343 LC and MPRL 1504 whole cells revealed dark-staining material in the high and intermediate *Mr* regions, but little in the low *Mr* region. The rabbit serum against the common Ag revealed only one band towards the top of the track. This band was also evident in the blot using the mouse MAb 6G3 (possibly anti-common Ag). Of the other mouse MAbs, 3C8 (anti-O-Ag), 3D7 (anti-LC and EDL populations) and 2E12 complexed with material at the top of the tracks, 3H5, 5B7, 1A4 (reactive with 99% of *B. fragilis* isolates) and 1E2 complexed with material slightly lower on the tracks, and 2B8 and 3C10 complexed with material slightly lower again. No staining was observed with MAb 3F6 (against an undefined epitope in the SC population). Immunoblotting of the high *Mr* material with polyclonal rabbit sera only revealed material when serum against NCTC 9343 LC and serum against NCTC 9344 whole cells were used. In both of these cases, the material was in the high *Mr* region of the gel. Rabbit serum against the common Ag, and the mouse MAb 6G3 did not reveal any material. Of the other mouse MAbs, 3C8 and 3D7 complexed with high *Mr* material, 3H5 and 3C10 complexed with material slightly lower on the tracks, and 2B8 complexed with material slightly lower again. None of the other MAbs revealed any material. The other fractions (PSA, PSB, intermediate *Mr* and low *Mr*) which were visible on certain dot blots were not visible by immunoblotting following PAGE.

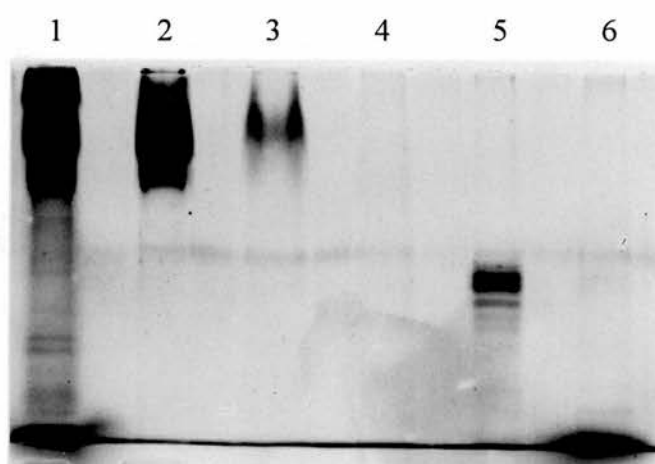


Figure 30. Silver-stained PAGE (8% acrylamide) profiles of an aqueous phenol extract and surface polysaccharide fractions of *B. fragilis* NCTC 9343 (20 μg / track). Tracks: **1**, whole aqueous phenol extract; **2**, high *Mr* material; **3**, PSA; **4**, PSB; **5**, intermediate *Mr* material; **6**, low *Mr* material.

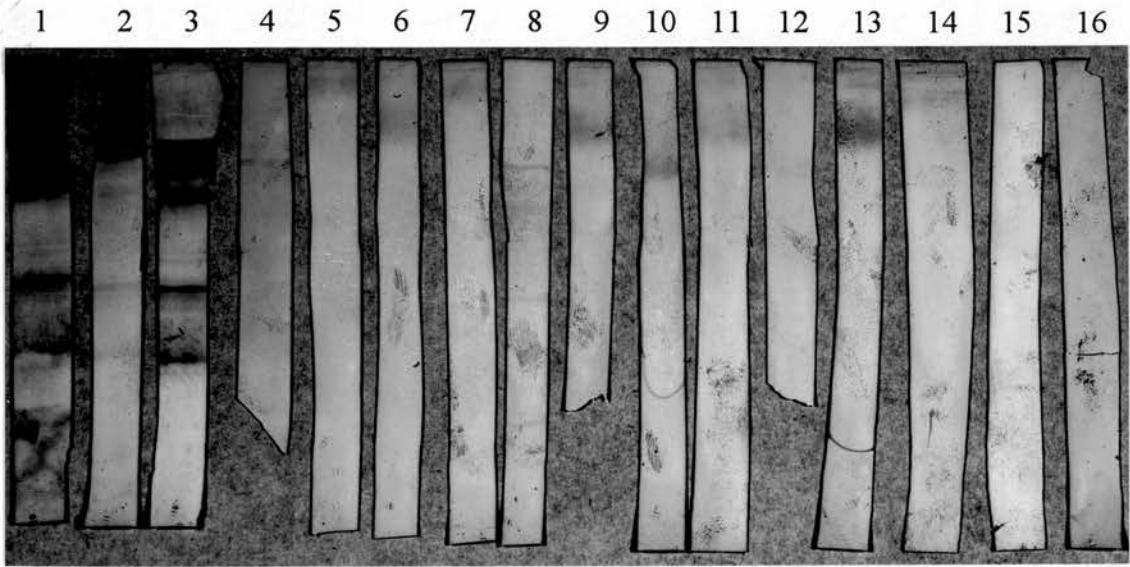


Figure 31a. Multiblots of polyacrylamide (8%) gel of a whole aqueous phenol extract of *B. fragilis* NCTC 9343 using polyclonal rabbit serum and murine MAbs. Tracks: Rabbit sera: **1**, against *B. fragilis* NCTC 9344 whole cells; **2**, against *B. fragilis* MPRL 1504 whole cells; **3**, against *B. fragilis* NCTC 9343 LC; **4**, against *B. fragilis* common Ag; Murine MAbs: **5**, 3C8; **6**, 3H5; **7**, 3D7; **8**, 6G3; **9**, 5B7; **10**, 2B8; **11**, 1A4; **12**, 3C10; **13**, 4C5; **14**, 1E2; **15**, 2E12; **16**, 3F6 (7 day supernate).

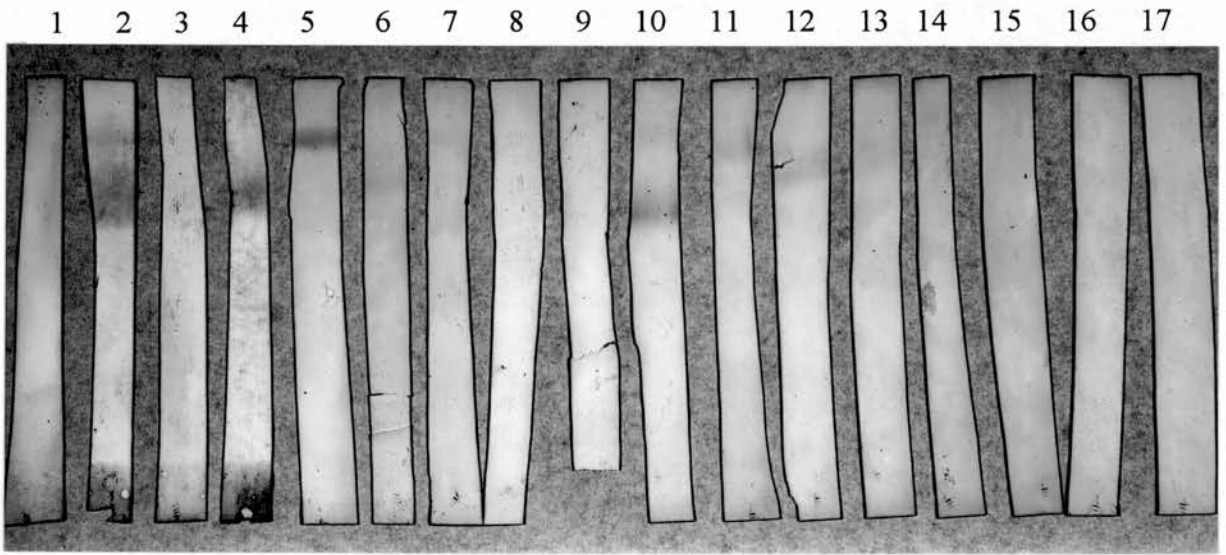


Figure 31b. Multiblots of polyacrylamide (8%) gel of the high *Mr* fraction of the surface polysaccharide complex of *B. fragilis* NCTC 9343, using polyclonal rabbit serum and murine MAbs. Tracks: Rabbit sera: 1, against *B. fragilis* MPRL 1504 whole cells; 2, against *B. fragilis* NCTC 9343 LC; 3, against *B. fragilis* common Ag; 4, against *B. fragilis* NCTC 9344; Murine MAbs: 5, 3C8; 6, 3H5; 7, 3D7; 8, 6G3; 9, 5B7; 10, 2B8; 11, 1A4; 12, 3C10; 13, 4C5; 14, 1E2; 15, 2E12; 16, 3F6 (2 day supernate); 17, 3F6 (7 day supernate).

3.4 ANTIBODIES TO BACTEROIDES IN HEALTH AND DISEASE

The aim of this study was to carry out a comprehensive investigation of IgG levels to bacteroides in serum, both in health and disease. IgG was chosen as its presence in serum indicates previous exposure to an Ag, and in disease a sudden consumption of IgG against a particular Ag may indicate re-exposure. Unless otherwise indicated, the conjugate used to detect serum Abs was an anti-human IgG (whole molecule) conjugated to alkaline phosphatase.

3.4.1 ANTIGENS USED FOR MEASURING IgG IN SERA

The Ags chosen to measure Ab levels against were non-ultracentrifuged aqueous phenol extracts of selected *Bacteroides* spp. (grown in PPY) and a cocktail of PCP-extracted LPS from *E. coli*, *S. typhimurium*, *K. pneumoniae* and *P. aeruginosa*. The silver-stained profiles of the different extracts are shown in Figure 32. The only striking difference between the large-scale and the rapid aqueous phenol extracts of the *Bacteroides* strains was in *B. thetaiotaomicron* NCTC 10582; the large-scale extract had several evenly spaced bands characteristic of smooth LPS above the low *Mr* material which were not apparent in the rapid extracts. The PCP-extracted LPSs were, as expected, all of the rough phenotype.

Although the bacteroides aqueous phenol extracts were not pure LPS, for the sake of ease in the rest of this section they will be referred to as LPS.

3.4.2 PRELIMINARY SCREEN OF 50 RANDOM BLOOD DONORS FOR ANTI-BACTEROIDES LIPOPOLYSACCHARIDE IgG

Initially 50 random blood donors were screened by ELISA for IgG against nine bacteroides LPSs. Scatter graphs were drawn by plotting the OD obtained from each donor serum with one Ag against the OD obtained from the same serum with another Ag. Correlation coefficients and P values were then calculated. The results for all of

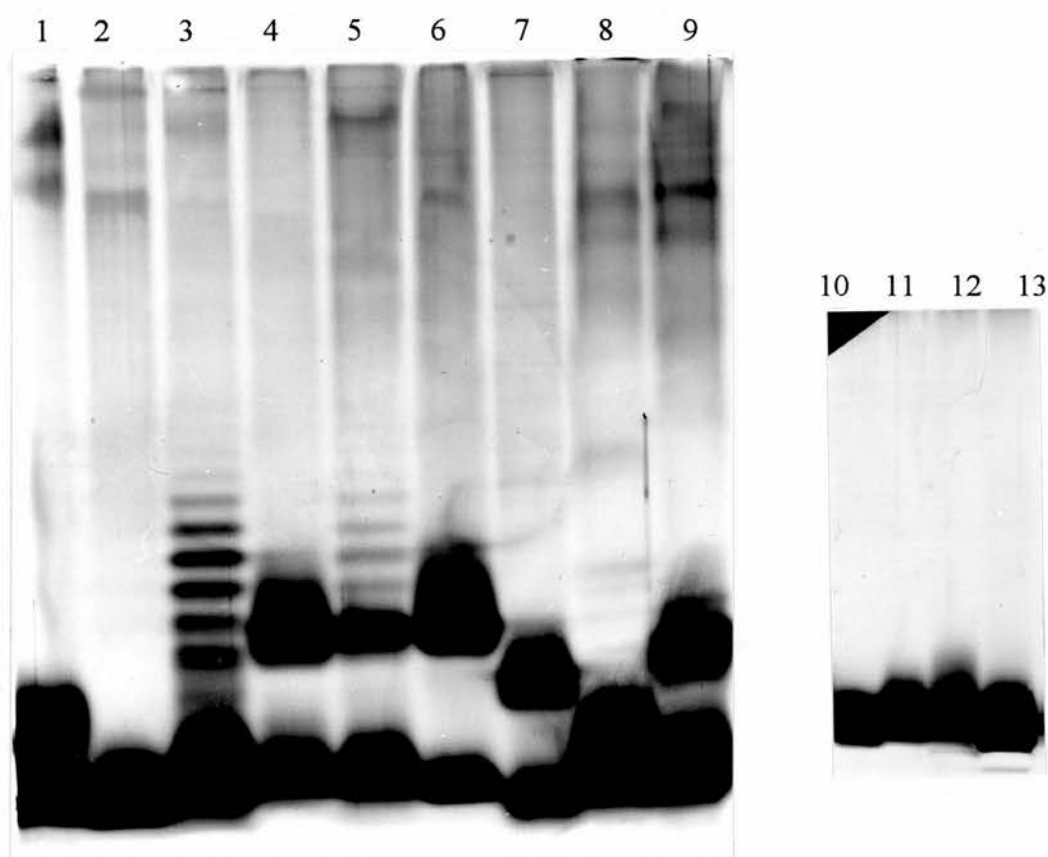


Figure 32. Silver-stained PAGE (14%) profiles of aqueous phenol extracts of selected *Bacteroides* strains and of PCP extracts of three enterobacteria and *P. aeruginosa* 20 μ g / track). Tracks: **1**, *B. fragilis* NCTC 9343; **2**, *B. fragilis* MPRL 1504; **3**, *B. vulgatus* MPRL 1985; **4**, *B. caccae* MPRL 1555; **5**, *B. thetaiotaomicron* NCTC 10582; **6**, *B. ovatus* MPRL 2370; **7**, *B. variabilis* VPI 11368; **8**, *B. eggerthii* NCTC 11155; **9**, *B. uniformis* ATCC 8492; **10**, *E. coli* K12 (MPRL 1303); **11**, *S. typhimurium* Rc 878 (MPRL 0947); **12**, *K. pneumoniae* 10B (MPRL 1954); **13**, *P. aeruginosa* PAC 611 (MPRL 1091).

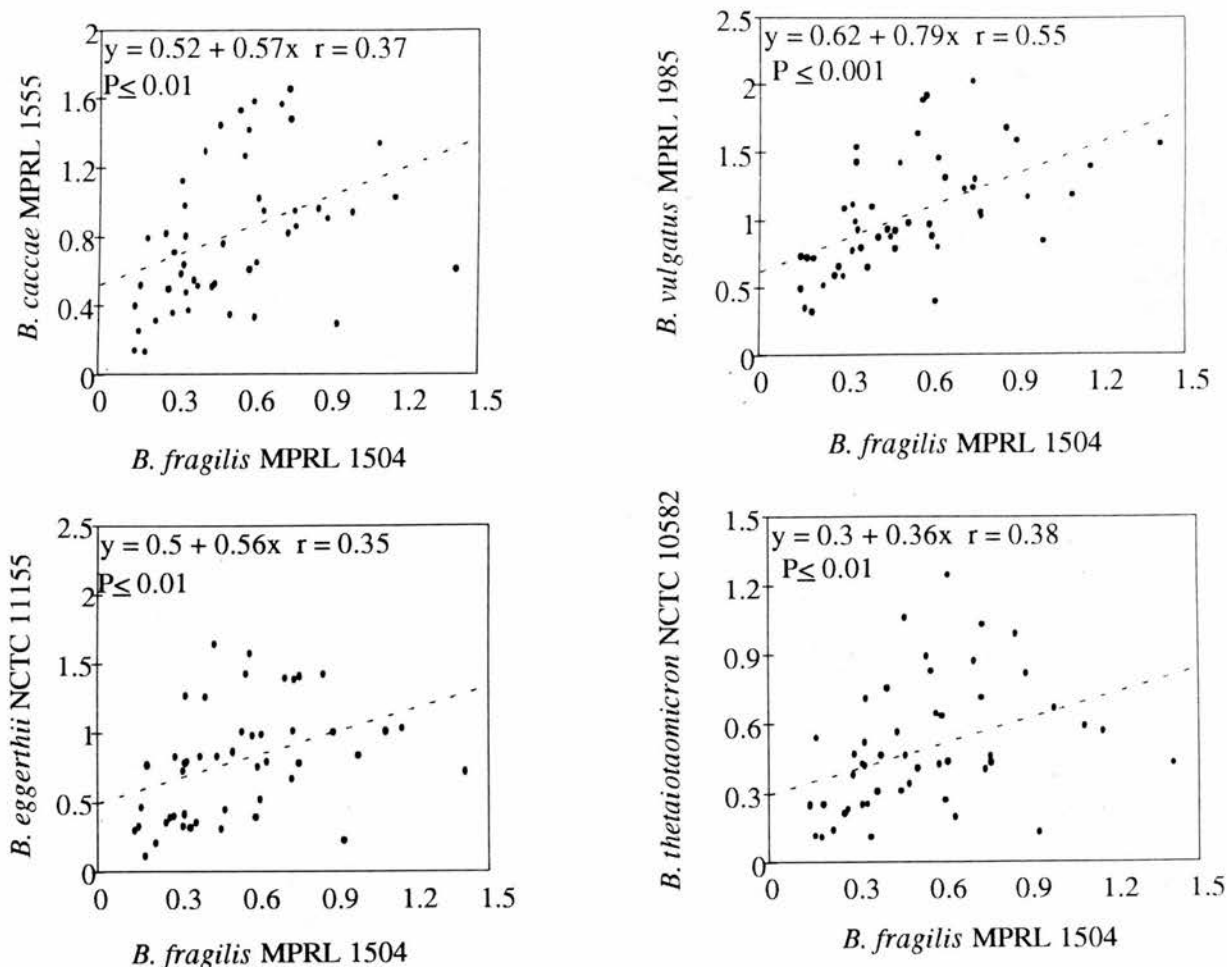


Figure 33. Scatter graphs showing relative IgG levels to one bacteroides aqueous phenol extract compared to IgG levels against another in 49 random blood donors, as measured by $OD_{405\text{ nm}}$ in ELISA (x and y axes). • = one donor; ---- = best fit line through points; y = y-axis intercept of best fit line; x = gradient of best fit line; r = correlation coefficient; P = probability of the correlation occurring by chance; NS = correlation not significant ($P \geq 0.05$).

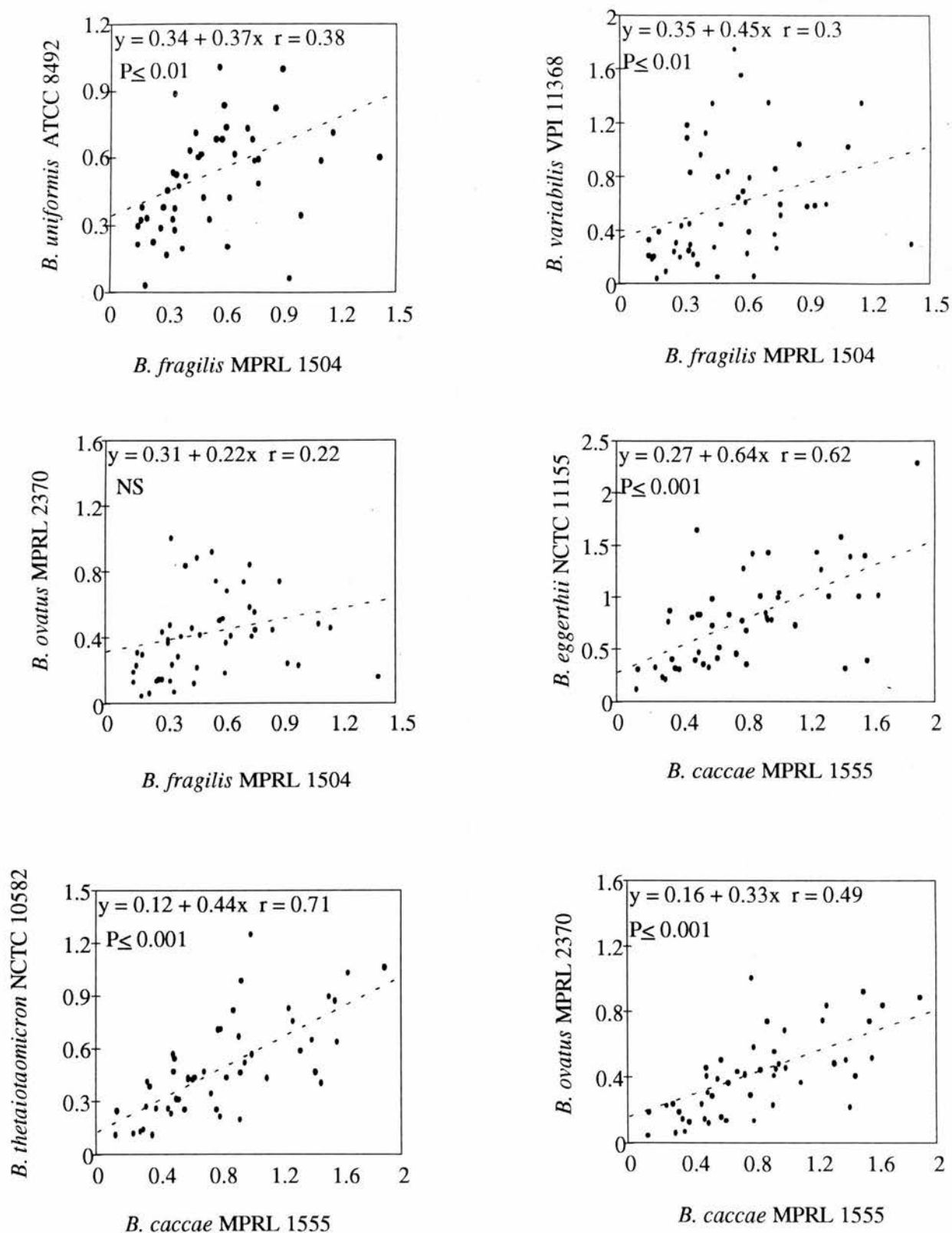


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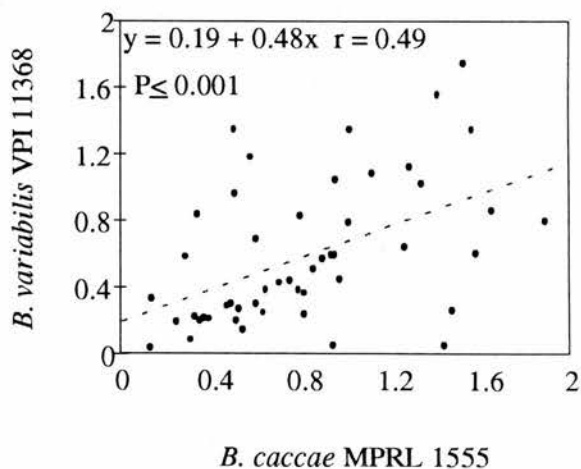
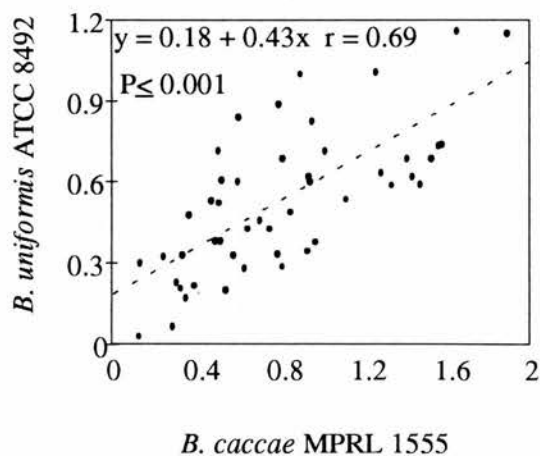
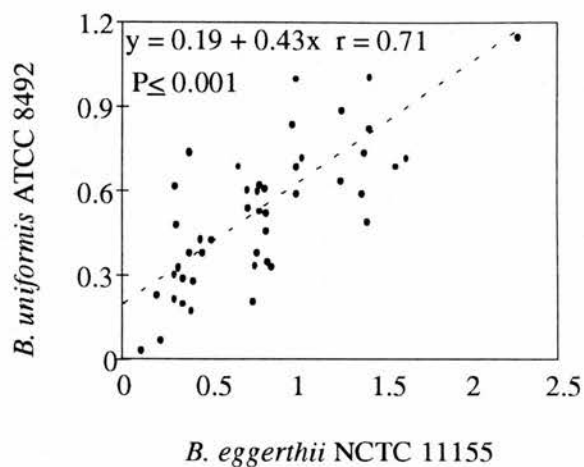
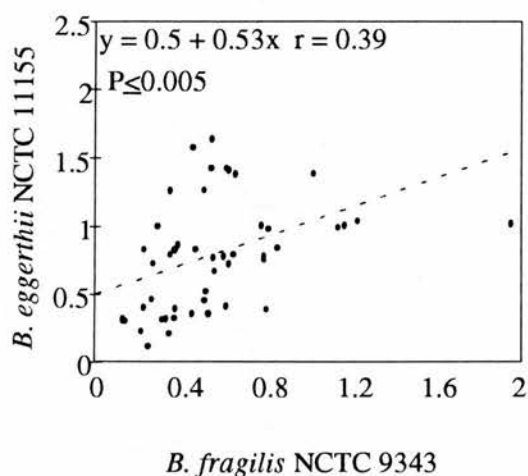
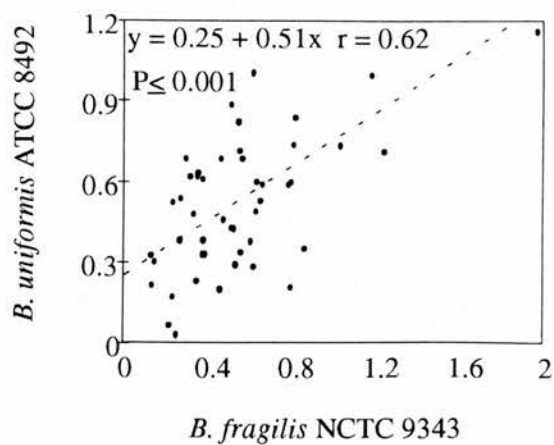
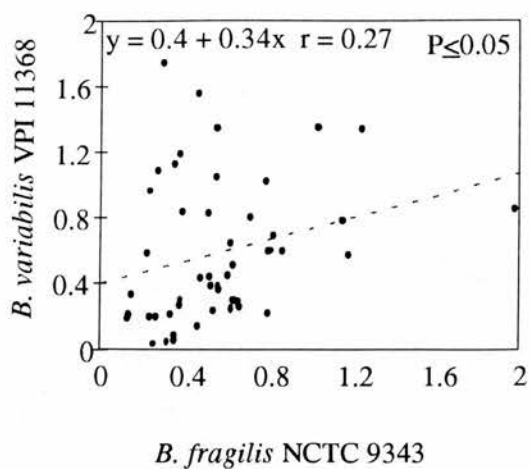


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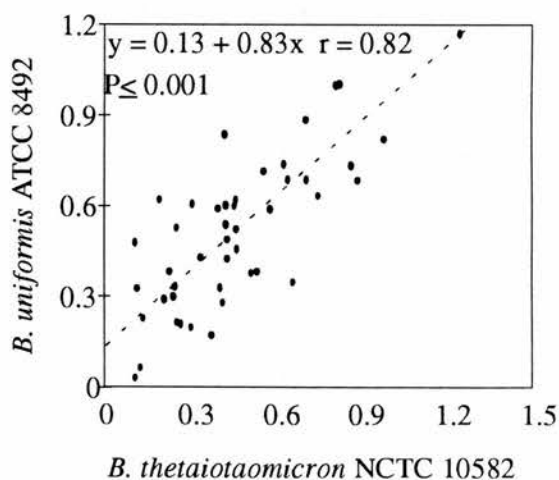
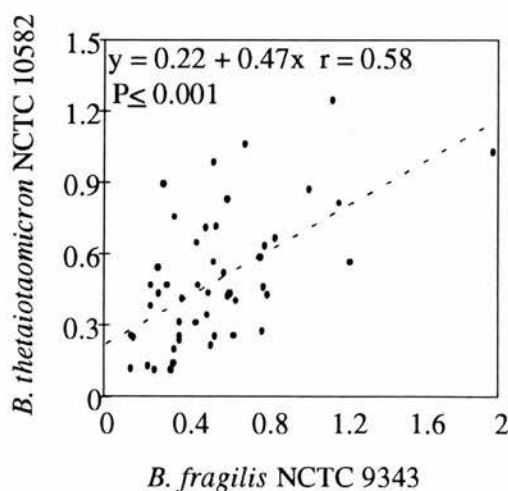
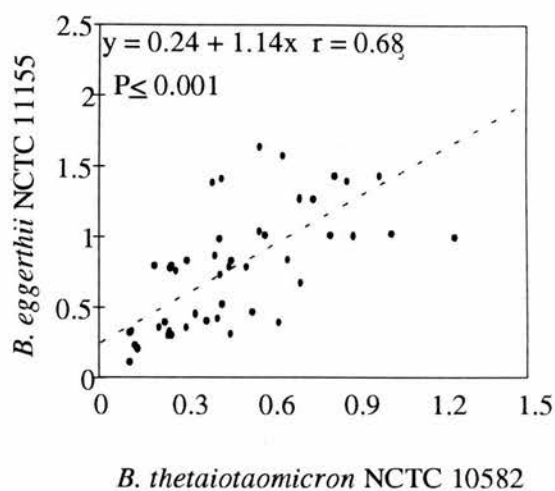
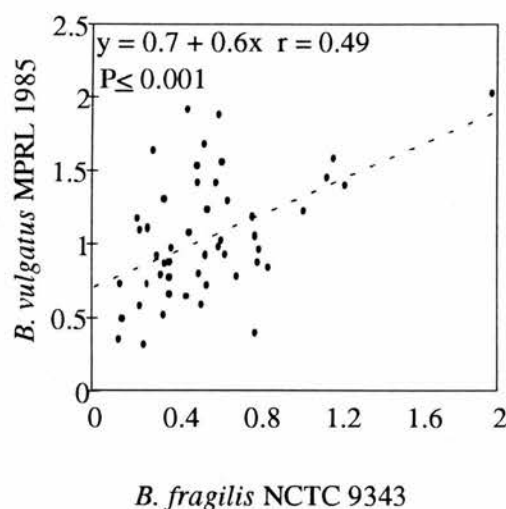
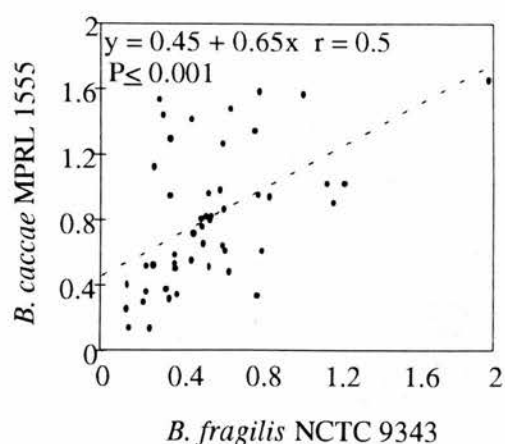
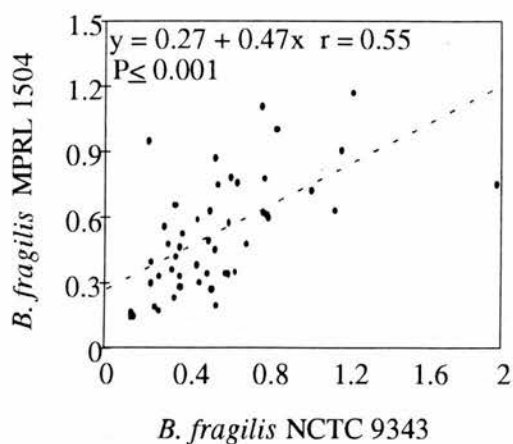


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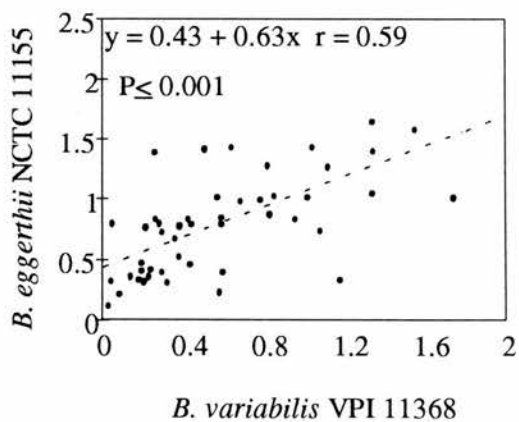
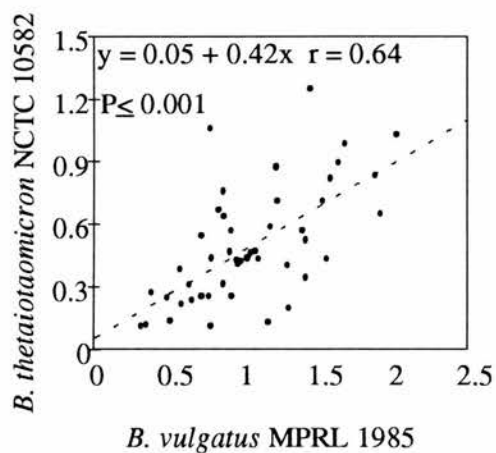
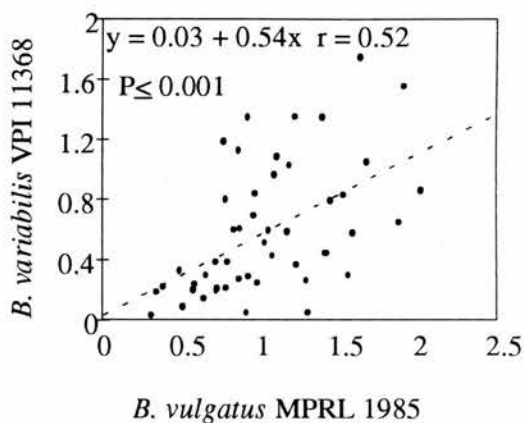
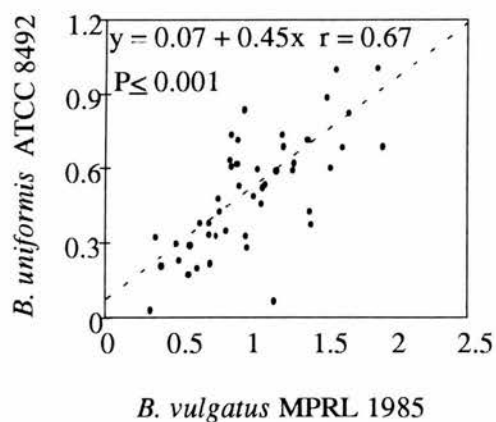
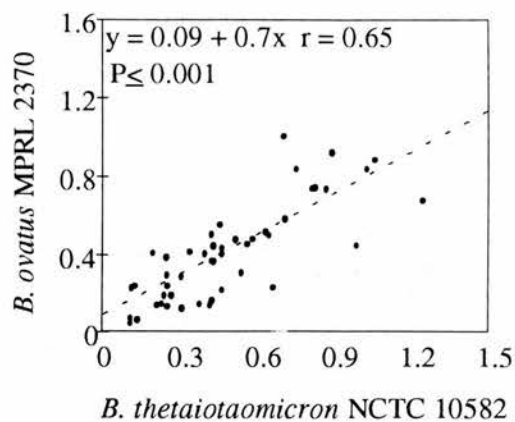
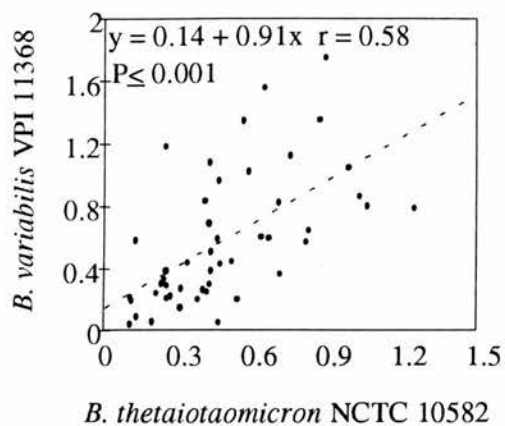


Figure 33 (continued).

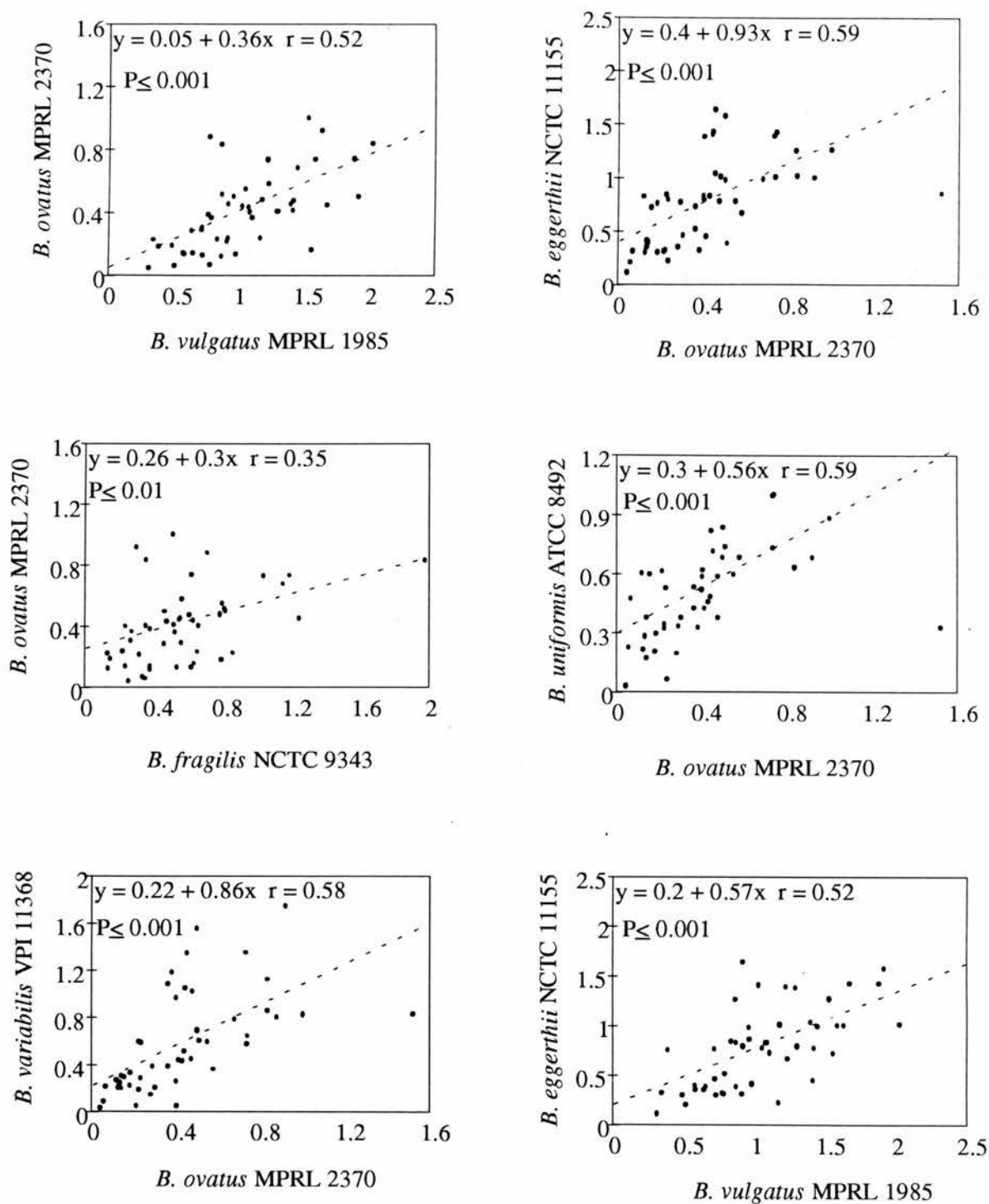


Figure 33 (continued).

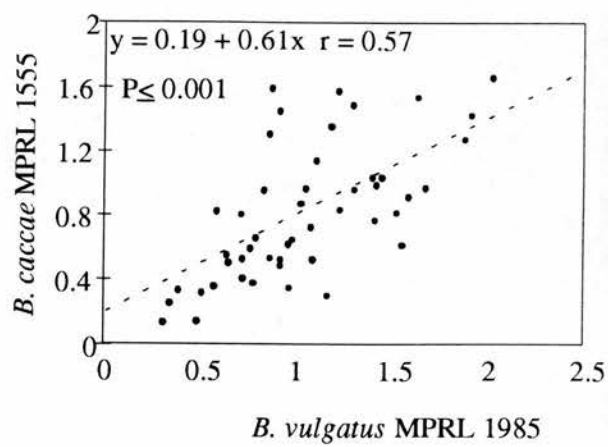
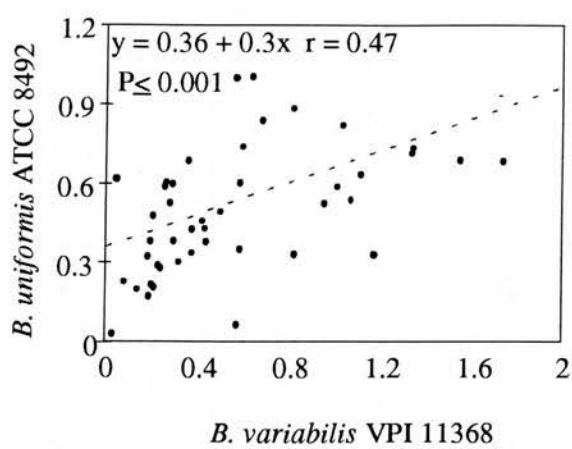


Figure 33 (continued).

the Ags are shown in Figure 33. There was a high degree of correlation between most Ags tested - i.e. in any given donor when the IgG levels to one Ag were high, the levels to the other Ag were correspondingly high and vice-versa. The only case where the correlation was not significant ($r = 0.22$; $P > 0.5$) was between *B. fragilis* MPRL 1504 and *B. ovatus* MPRL 2370. Interestingly, the only cases where correlation coefficients were not very highly significant (≥ 0.4 ; $P \leq 0.001$) were between the *B. fragilis* strains and some of the other strains.

This study showed that in virtually all cases there was a significant correlation between the IgG levels to LPSs from different species and strains of *Bacteroides*. There were two different ways in which this could have occurred:

- The Abs cross-reacted between different *Bacteroides* species.
- The Abs were not cross-reactive, but some individuals were exposed to bacteroides Ags more than others, so when the response to one was high, the response to another was also correspondingly high.

In an attempt to find out how cross-reactive the anti-bacteroides Abs were, absorption studies were carried out.

3.4.3 ABSORPTION OF ANTI-BACTEROIDES LIPOPOLYSACCHARIDE ANTIBODIES USING WHOLE CELLS

Serum pooled from ten random blood donors was subjected to a series of absorptions with bacteroides whole cells to absorb out specific Abs. It was then used in an ELISA measuring IgG levels to the different LPSs; a decrease in IgG levels compared to a non-preabsorbed control would indicate the presence in serum of cross-reactive Abs. The results of this are shown in Figure 34. To standardise results

so that they could all be similarly compared, ODs were converted to a percentage of a non-preabsorbed control (which was given a value of 100%).

In the majority of cases, preabsorption of serum with bacteroides whole cells reduced the subsequent amount of IgG bound to any given LPS in ELISA. The degree of reduction was highly dependent on which LPS and cells were used.

Generally, binding of IgG to the different LPSs was reduced to the greatest extent when serum had been preabsorbed with whole cells of the same strain from which the LPS was derived. There was one exception to this: binding of IgG to *B. eggerthii* NCTC 11155 LPS was reduced almost equally following preabsorption of serum with *B. eggerthii*, *B. vulgatus* and *B. thetaiotaomicron* whole cells.

The extent by which preabsorption of serum with whole cells reduced IgG binding to LPS derived from the same cells was variable between different species and strains. Preabsorption of serum with *B. fragilis* NCTC 9343 and *B. vulgatus* MPRL 1985 whole cells reduced the subsequent amount of IgG bound to *B. fragilis* NCTC 9343 and *B. vulgatus* MPRL 1985 LPS respectively by about 90%. On the other hand, preabsorption of serum with *B. eggerthii* NCTC 11155 and *B. thetaiotaomicron* NCTC 10582 reduced the subsequent amount of IgG bound to the respective LPSs by only 50-60%.

In most cases, a certain degree of cross-reactivity was apparent, but certain Abs appeared to be more cross-reactive than others, suggesting that LPSs of certain strains shared more common epitopes than others. There was a high degree of cross-reactivity between IgG against the two *B. fragilis* strains, and between IgG against *B. vulgatus* MPRL 1985, *B. thetaiotaomicron* NCTC 10582 and *B. variabilis* VPI 11368. Binding of IgG to *B. uniformis* ATCC 8492 LPS was reduced by at least

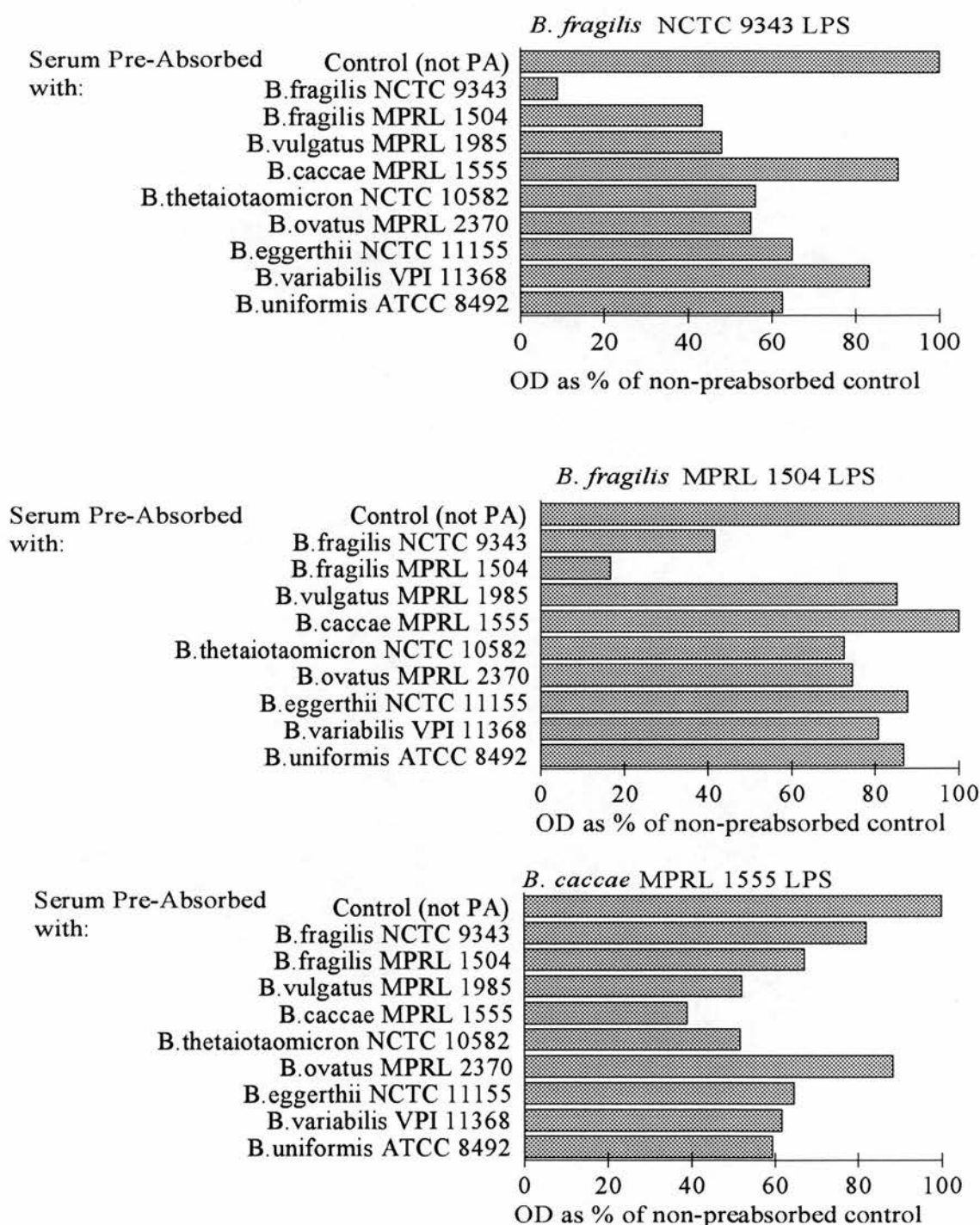


Figure 34. Measurement of IgG levels to bacteroides aqueous phenol-extracted LPS in ELISA following preabsorption of serum with bacteroides whole cells. OD (405 nm) is expressed as a percentage of a non-preabsorbed control. PA = preabsorbed.

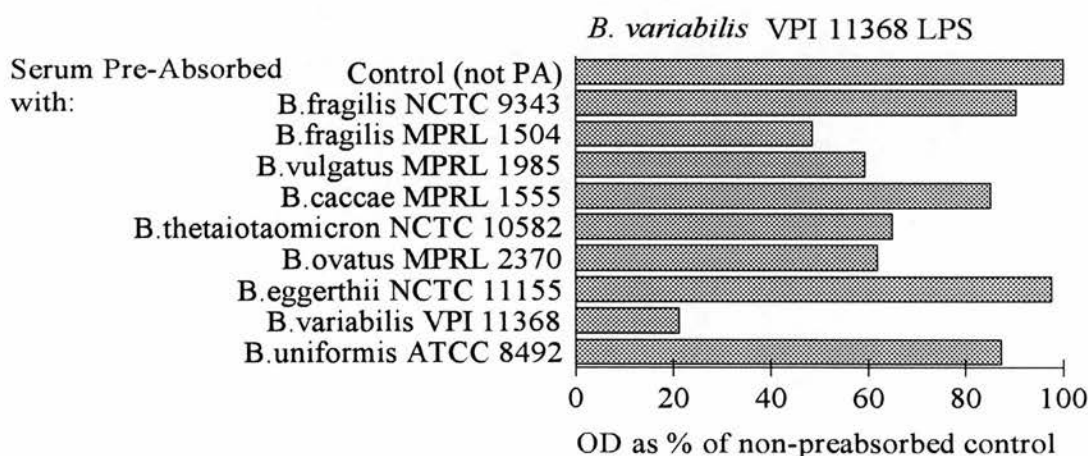
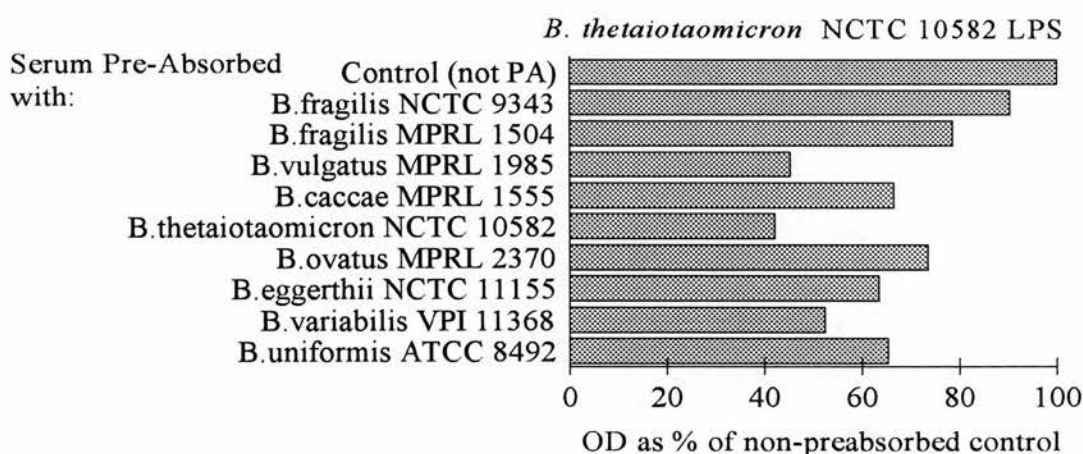
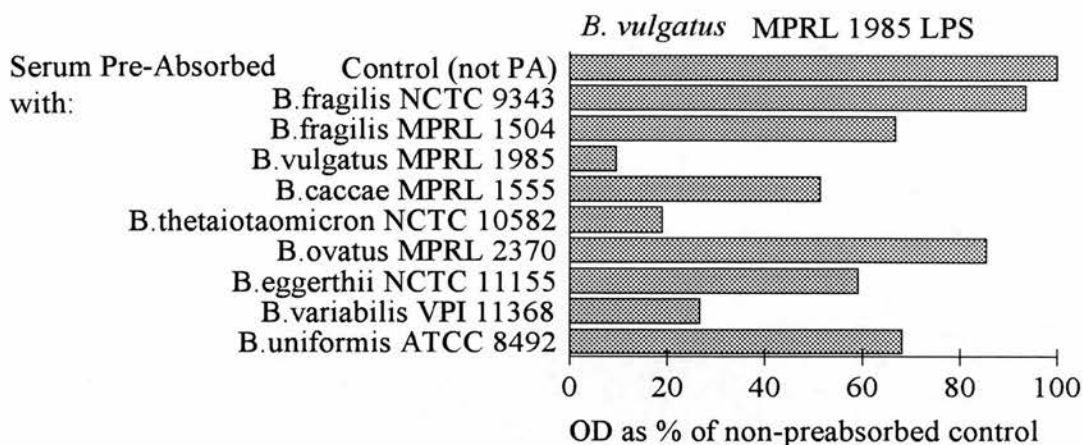


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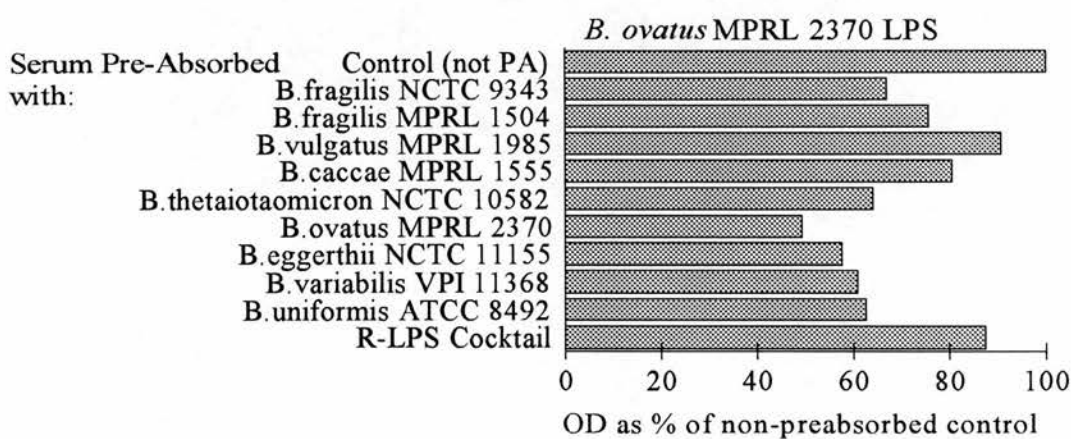
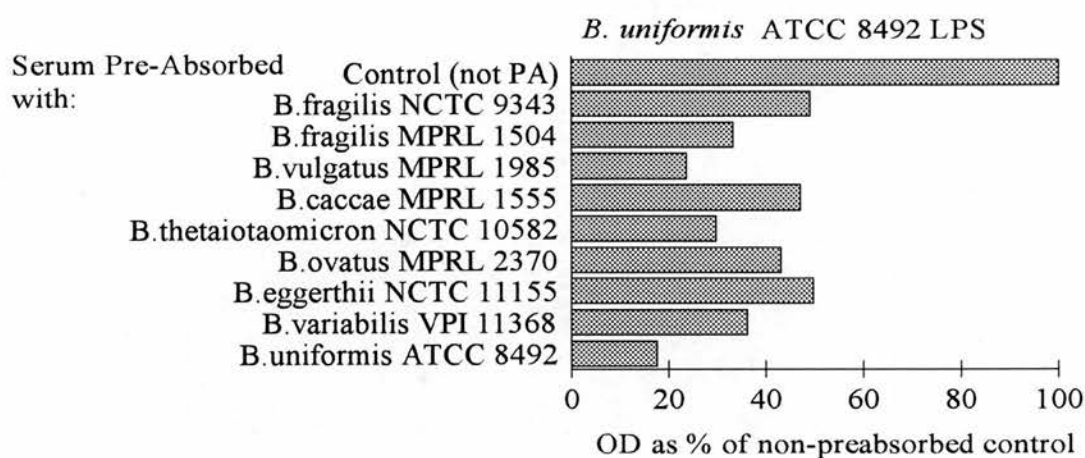
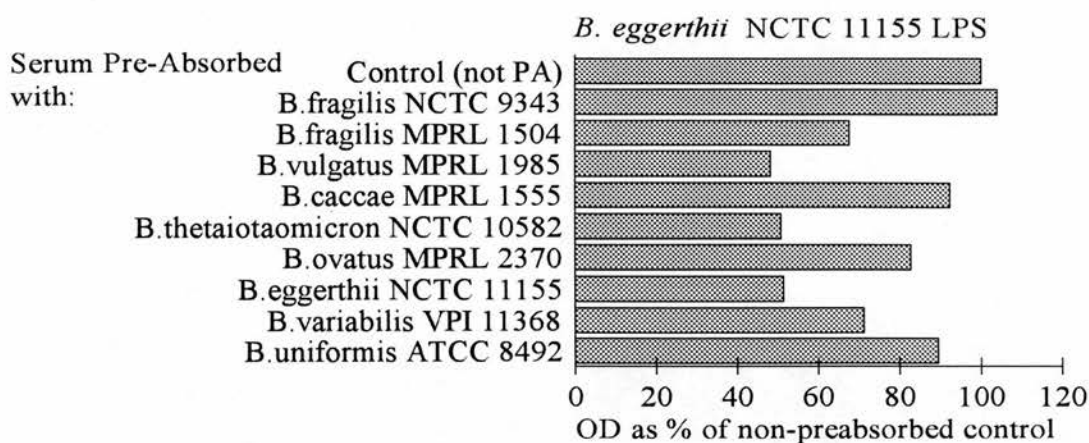


Figure 34 (continued).

50% in all cases following preabsorption of serum, suggesting that the LPS of this strain possibly had an epitope common to all strains tested.

In summary, it appeared that there were some epitopes common to all of the bacteroides LPSs, but in addition each strain also possessed unique epitopes with distinctive Abs against them present in serum.

A problem with using whole cells to try to absorb out cross-reactive Abs is that certain epitopes (e.g. lipid A) are masked on whole cells, and so Abs against these epitopes are not absorbed out. To try to overcome this problem, Abs were absorbed out of serum using LPS rather than whole cells, prior to using the serum in ELISA.

3.4.4 ABSORPTION OF ANTI-BACTEROIDES LIPOPOLYSACCHARIDE ANTIBODIES USING LIPOPOLYSACCHARIDE

For these experiments, the same nine bacteroides LPSs, plus the enterobacterial / *Pseudomonas* R-LPS cocktail, were used in the ELISA to measure serum IgG. However, only four bacteroides LPSs were selected to absorb Abs from serum in order to make the execution and interpretation of the experiment more manageable. LPSs used were from *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 (as *B. fragilis* is the most common anaerobic pathogen), *B. thetaiotaomicron* NCTC 10582 and *B. vulgatus* MPRL 1985 (as these are two of the most common *Bacteroides* spp. in faeces) and the enterobacterial / *Pseudomonas* R-LPS cocktail (in order to assess degree of cross-reactivity between bacteroides and enterobacterial LPS). In addition, to compare Ab responses in pooled serum with those in an individual's serum, and to compare the Ab response between different individuals, absorptions and ELISAs were carried out with serum pooled from ten random donors and with serum from two individual volunteers. The results (shown in Figure 35a-e) were standardised as before.

The trends and patterns of cross-reactivity were generally the same as those seen with whole cell absorptions. There were some epitopes on the different bacteroides LPSs that were common, but also others that were unique to each particular strain. *B. vulgatus* MPRL 1985 and *B. thetaiotaomicron* NCTC 10582 LPS appeared to share many common epitopes, as was observed in whole cell absorptions; however, IgG against the two *B. fragilis* LPSs appeared to show less cross-reactivity when serum was preabsorbed with LPS as opposed to whole cells.

Although overall trends were the same, there were some differences between degrees of cross-reactivity of anti-bacteroides LPS IgG between the pooled serum and the two individuals' sera. For example, the pooled donor serum had more anti-*B. fragilis* NCTC 9343 LPS IgG which cross-reacted with *B. eggerthii* NCTC 11155 LPS than either of the two individuals, and individual # 2 had a greater amount of anti-*B. fragilis* NCTC 9343 LPS IgG which cross-reacted with all of the other bacteroides LPSs than the pooled serum or than individual # 1. This suggested that the anti-bacteroides Ab repertoire varied between different individuals, and the cross-reactivity pattern seen in the pooled serum was the cumulative result of the IgG repertoires in the ten individuals' sera.

In all cases, binding of IgG to the different LPSs was reduced to the greatest extent when serum had been preabsorbed with homologous LPS. As with the whole cell absorptions, the extent to which preabsorption of serum with LPS reduced subsequent IgG binding to the same LPS was variable between different species and strains, and between different sera; preabsorption of pooled donor serum with *B. vulgatus* MPRL 1985 LPS reduced subsequent IgG binding to *B. vulgatus* MPRL 1985 by over 95%, whereas in individual # 1 the binding was only reduced by approximately 60% in the same experiment. Little difference was observed in the amount of cross-reactive Abs absorbed out when LPS was used for the absorptions as opposed to whole cells. Total

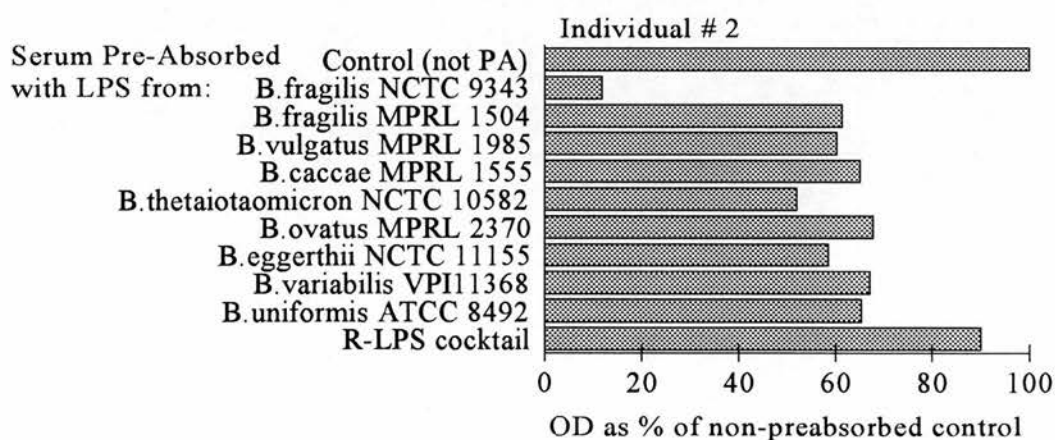
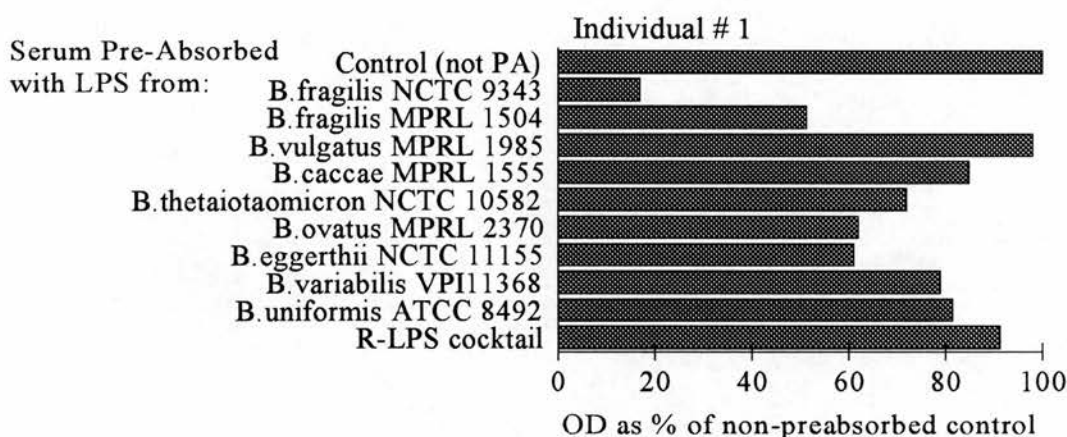
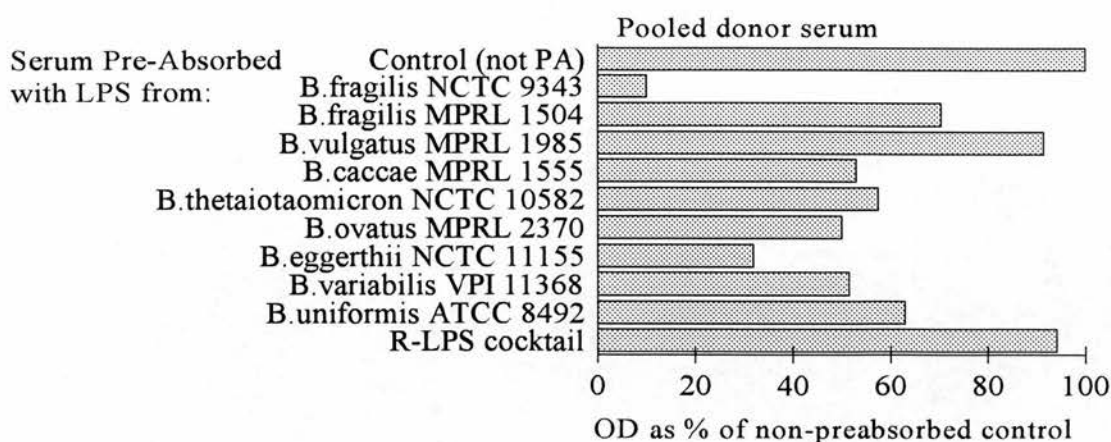


Figure 35a. Measurement of IgG levels to *B. fragilis* NCTC 9343 aqueous phenol-extracted LPS in ELISA following preabsorption of pooled and individual sera with bacteroides LPS and a cocktail of PCP-extracted R-LPS from three enterobacteria and *P. aeruginosa*. PA = preabsorbed. Results shown are means calculated from four replicates.

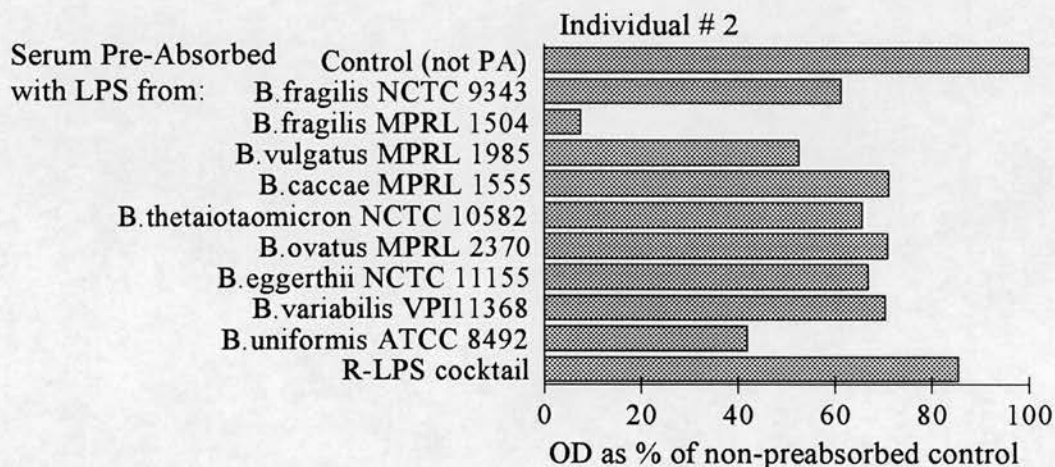
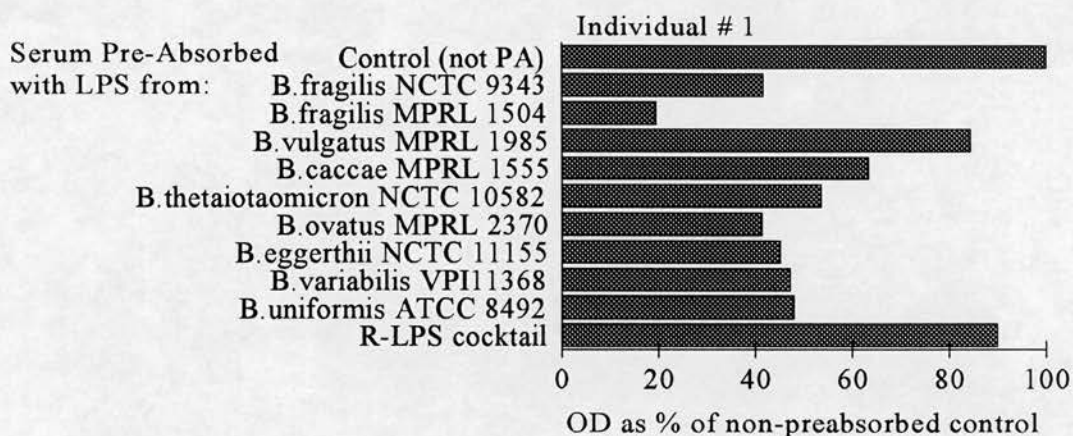
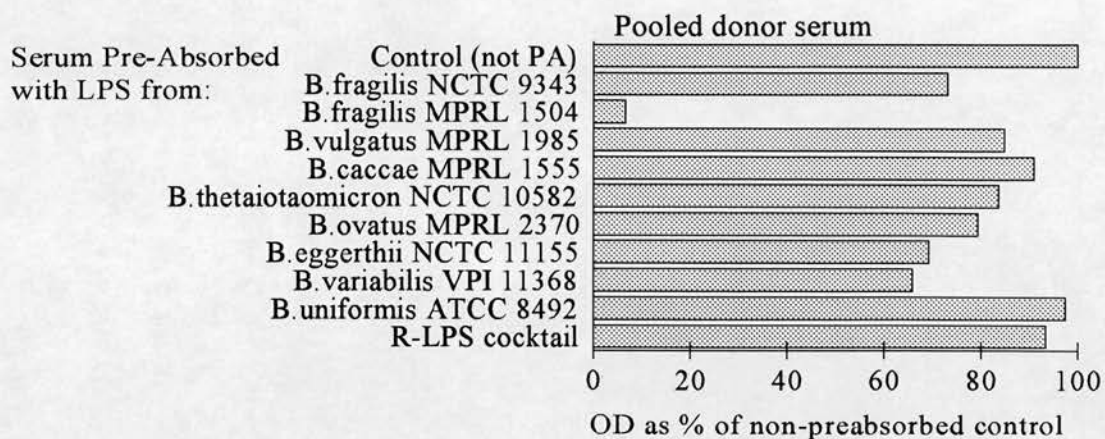


Figure 35b. Measurement of IgG levels to *B. fragilis* MPRL 1504 aqueous phenol-extracted LPS in ELISA following preabsorption of pooled and individual sera with bacteroides LPS and a cocktail of PCP-extracted R-LPS from three enterobacteria and *P. aeruginosa*. PA = preabsorbed. Results shown are means calculated from four replicates.

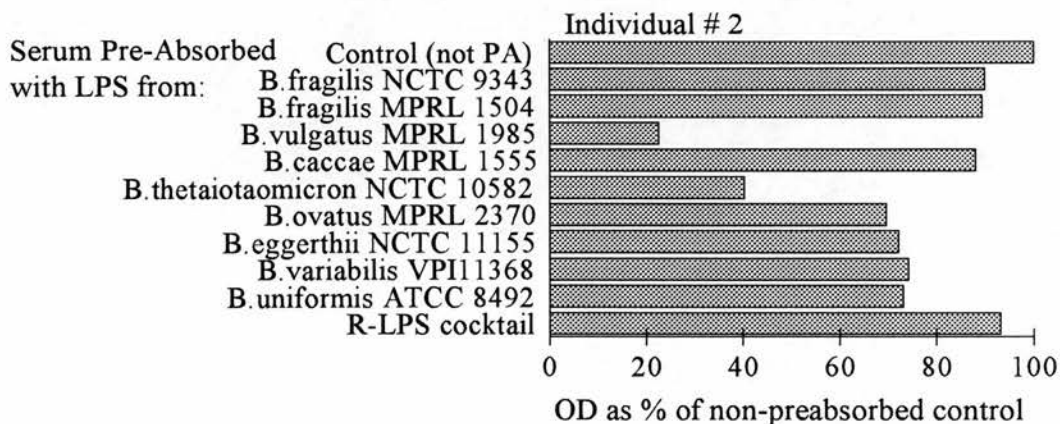
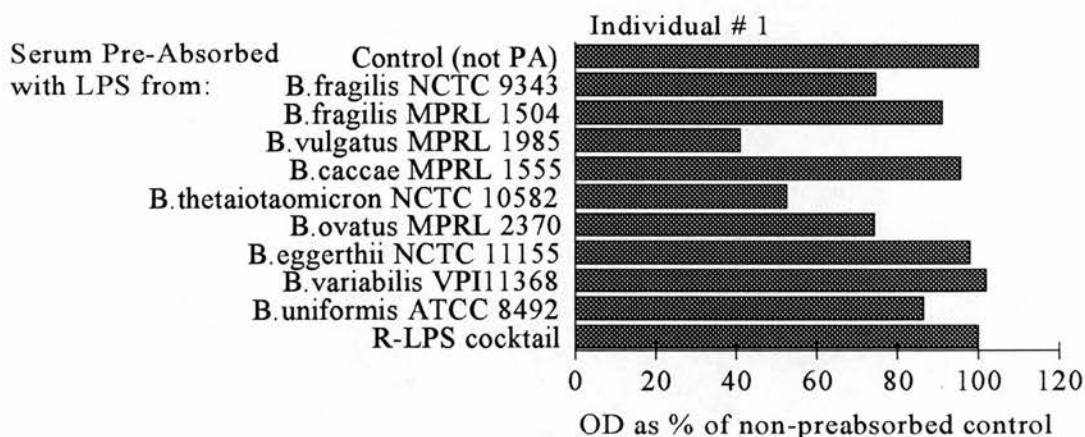
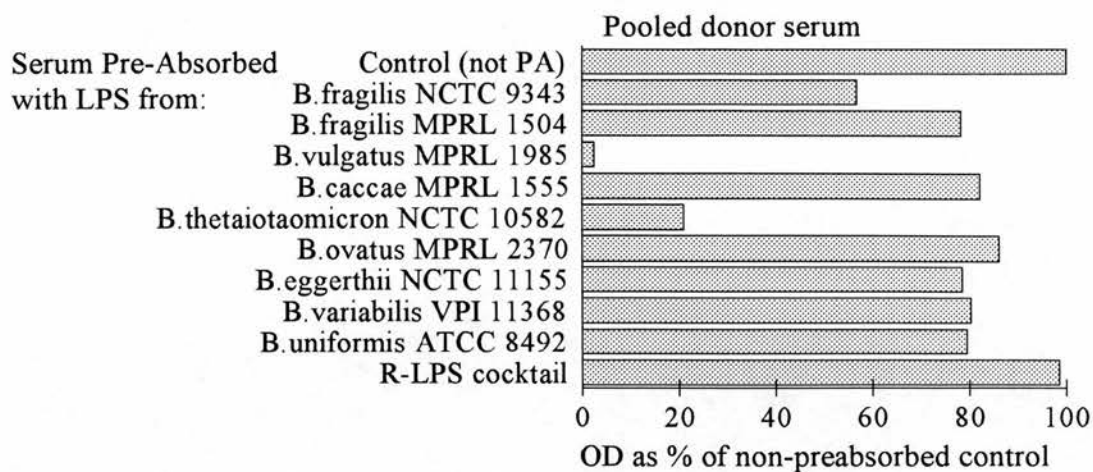


Figure 35c. Measurement of IgG levels to *B. vulgatus* MPRL 1985 aqueous phenol-extracted LPS in ELISA following preabsorption of pooled and individual sera with bacteroides LPS and a cocktail of PCP-extracted R-LPS from three enterobacteria and *P. aeruginosa*. PA = preabsorbed. Results shown are means calculated from four replicates.

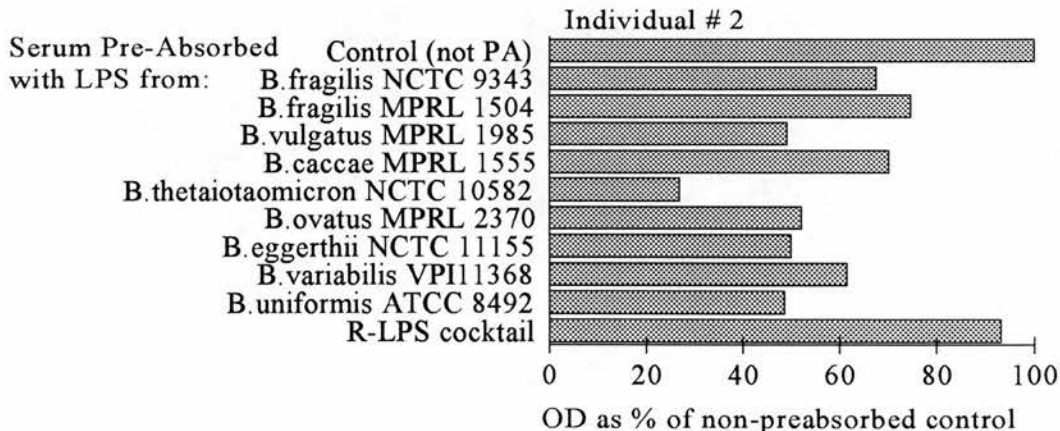
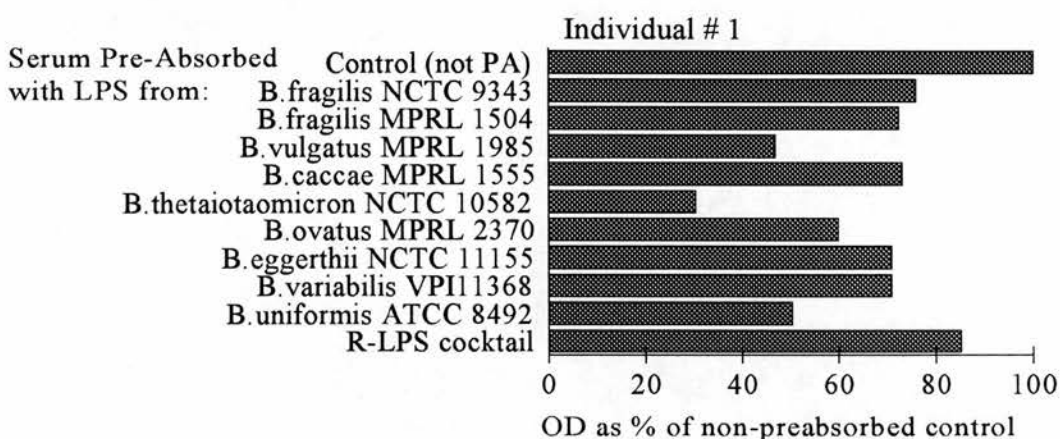
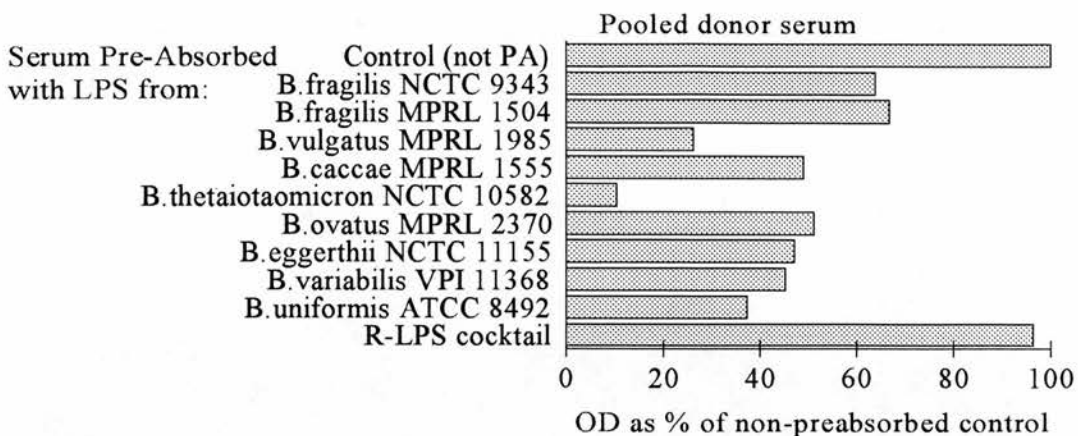


Figure 35d. Measurement of IgG levels to *B. thetaiotaomicron* NCTC 10582 aqueous phenol-extracted LPS in ELISA following preabsorption of pooled and individual sera with bacteroides LPS and a cocktail of PCP-extracted R-LPS from three enterobacteria and *P. aeruginosa*. PA = preabsorbed. Results shown are means of four replicates.

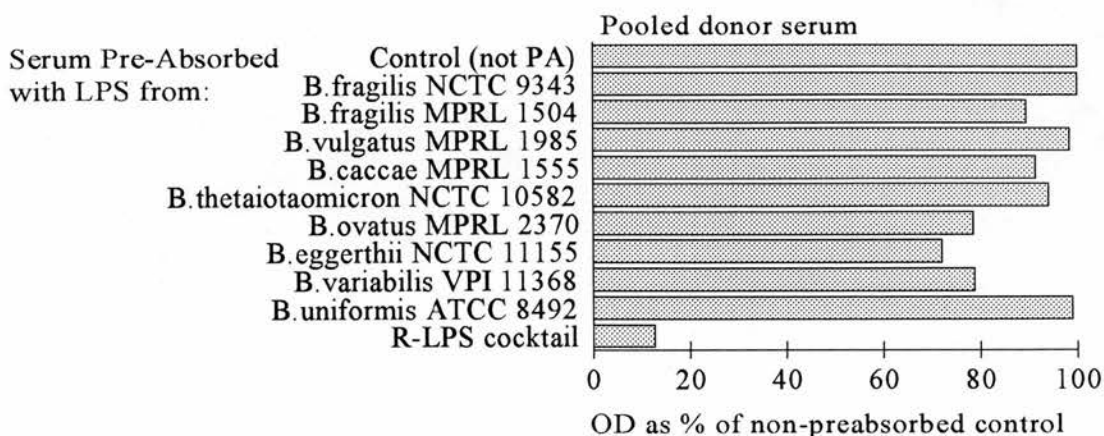


Figure 35e. Measurement of IgG levels to a cocktail of PCP-extracted R-LPS from three enterobacteria and *P. aeruginosa* in ELISA following preabsorption of pooled and individual sera with bacteroides LPS and the R-LPS cocktail. PA = preabsorbed. Results shown are means calculated from four replicates.

absorption of cross-reactivity was not achieved in any case.

The results of these experiments clearly showed that the degree of cross-reactivity between IgG against the rough enterobacterial / *Pseudomonas* LPS cocktail and IgG against bacteroides LPS was very limited. This suggests that there were very few common epitopes between the enterobacterial LPS and bacteroides LPS.

3.4.5 IMMUNOBLOTTING OF BACTEROIDES LIPOPOLYSACCHARIDES USING HUMAN SERUM

The aqueous phenol-extracted bacteroides LPS samples were examined by PAGE and immunoblotting to try to determine the epitopes to which the IgG was binding. Sera from the two individuals used above in the LPS absorption studies were used. The resultant immunoblots are shown in Figure 36a-b. In most cases, the LPS samples were not visualised by immunoblotting with human serum. In those that were, some differences were apparent between the two individuals. IgG from both individuals reacted with the smooth LPS from *B. vulgatus* and *B. thetaiotaomicron*, and with high *Mr* material from *B. caccae* and *B. ovatus* at the interface between the stacking and separating gels. However, in addition IgG from individual # 2 also reacted with low *Mr* material from *B. fragilis* NCTC 9343, high *Mr* material from *B. fragilis* MPRL 1504, low *Mr* material from *B. uniformis*, and with *B. eggerthii*, where a fine ladder pattern high in the smooth LPS region was apparent.

3.4.6 DETERMINATION OF THE DISTRIBUTION OF ANTI-LIPOPOLYSACCHARIDE IgG LEVELS WITHIN 641 BLOOD DONORS

Extensive studies have been previously carried out which have investigated distributions of Ab levels in human serum to enterobacteria and closely related spp. In contrast, to date there has been very little equivalent work carried out on *Bacteroides* spp. The aim of this study was therefore to look at the distribution of

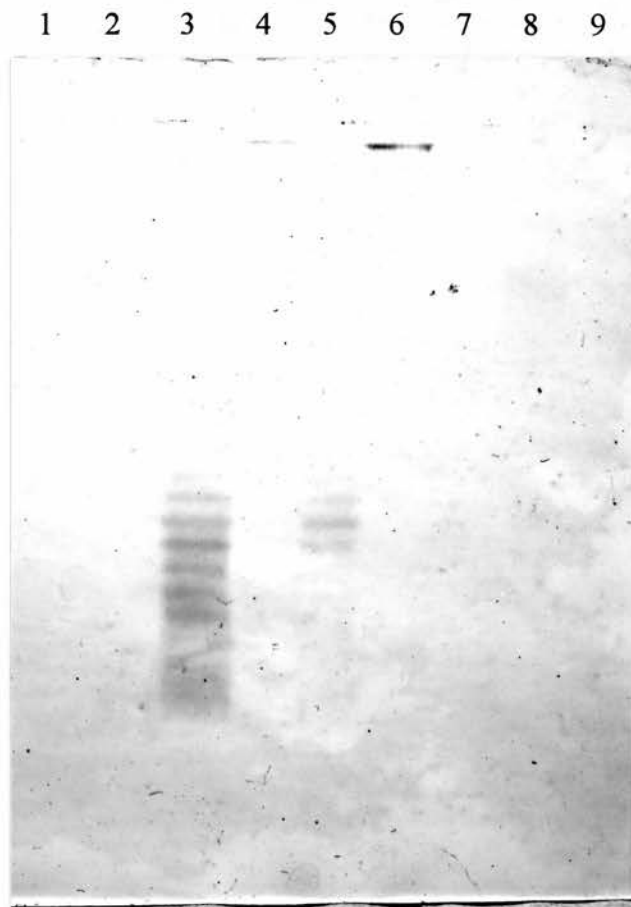


Figure 36a. Immunoblots of polyacrylamide (14%) gel of aqueous phenol-extracted bacteroides LPS (200 μ g / track), using human serum from **individual # 1** diluted 1 in 10. Tracks: **1**, *B. fragilis* NCTC 9343; **2**, *B. fragilis* MPRL 1504; **3**, *B. vulgatus* MPRL 1985; **4**, *B. caccae* MPRL 1555; **5**, *B. thetaiotaomicron* NCTC 10582; **6**, *B. ovatus* MPRL 2370; **7**, *B. variabilis* VPI 11368; **8**, *B. eggerthii* NCTC 11155; **9**, *B. uniformis* ATCC 8492.

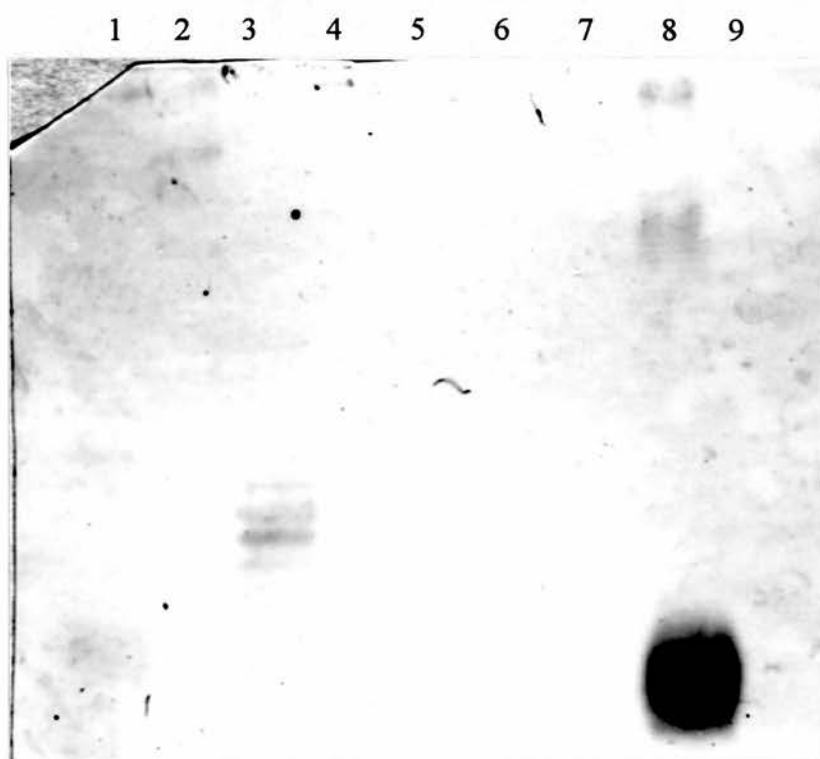


Figure 36b. Immunoblots of polyacrylamide (14%) gel of aqueous phenol-extracted bacteroides LPS (200 µg / track), using human serum from **individual # 2** diluted 1 in 10. Tracks as in Figure 36a.

anti-bacteroides LPS IgG in a large sample of blood donors, and to compare the distribution with that of anti-enterobacterial IgG. Sera from 641 blood donors were screened for IgG against aqueous phenol-extracted LPS from *B. fragilis* NCTC 9343, *B. vulgatus* MPRL 1985, *B. thetaiotaomicron* NCTC 10582 and the R-LPS cocktail (three enterobacteria and *P. aeruginosa*). The results of this screen are shown in Figure 37. Distributions of anti-LPS IgG in the donor population tested were normal in appearance on histograms, except anti-*B. fragilis* NCTC 9343 LPS IgG which showed a slight negative skew. Every donor screened had anti-bacteroides LPS IgG and anti-R-LPS cocktail IgG present in their serum.

3.4.7 KINETICS OF ANTI-LIPOPOLYSACCHARIDE IgG RESPONSE IN SYSTEMIC INFLAMMATORY RESPONSE SYNDROME PATIENTS

Previous studies have investigated the magnitude and kinetics of serum Ab response to LPS core from a range of enterobacteria and closely related spp. in SIRS patients. Ab levels, particularly IgG, were found to fluctuate considerably from day to day, and in some cases a sudden decrease in serum IgG was shown to be concurrent with an acute episode of endotoxaemia (Barclay *et al* 1989, Barclay 1990). No such study has been carried out with *Bacteroides* spp. The aim of this study was therefore to look at the kinetics of the IgG response to bacteroides LPS, and compare it with the kinetics of the response to LPS from enterobacteria and related spp. in SIRS patients. Patients selected for this study had a diagnosis of abdominal sepsis and were blood culture negative, and so any LPS present in their blood should have been derived from the gut. LPSs selected for the study (for the same reasons described in Section 3.4.4) were aqueous phenol extracts from *B. fragilis* NCTC 9343, *B. vulgatus* MPRL 1985, *B. thetaiotaomicron* NCTC 10582 and the R-LPS cocktail from three enterobacteria and *P. aeruginosa*. ELISAs were performed on sera taken daily over five- to nine-day periods from 12 SIRS patients (six survivors, six non-survivors). In addition, as a control, serum taken from a healthy individual over six consecutive

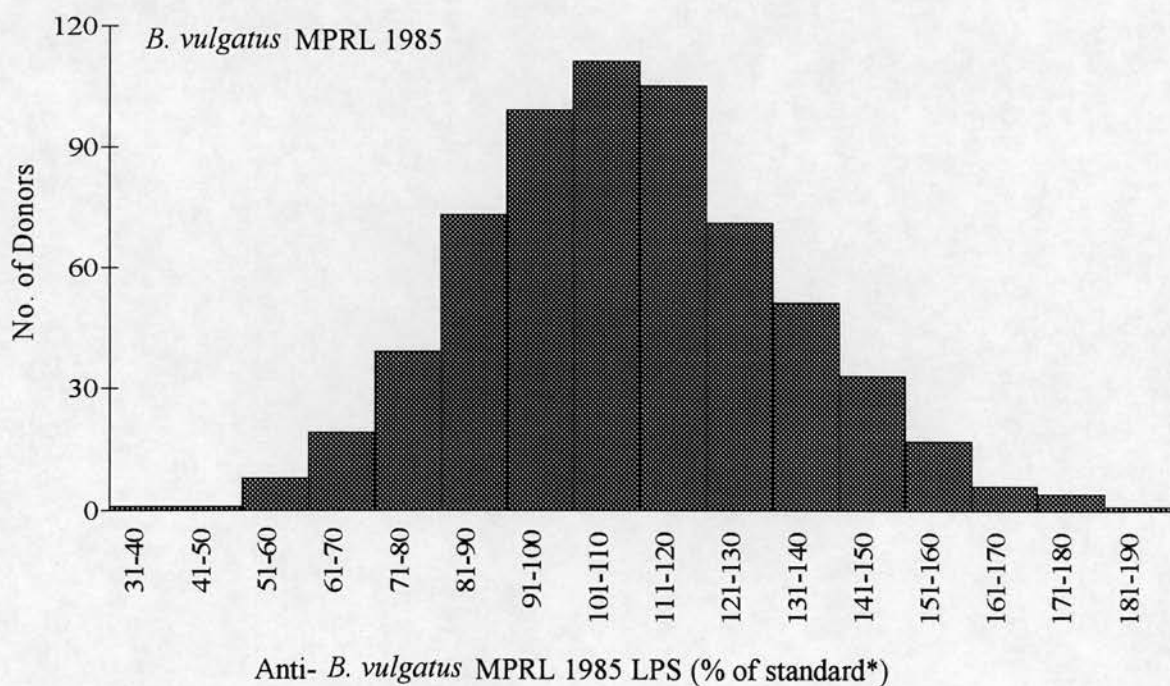
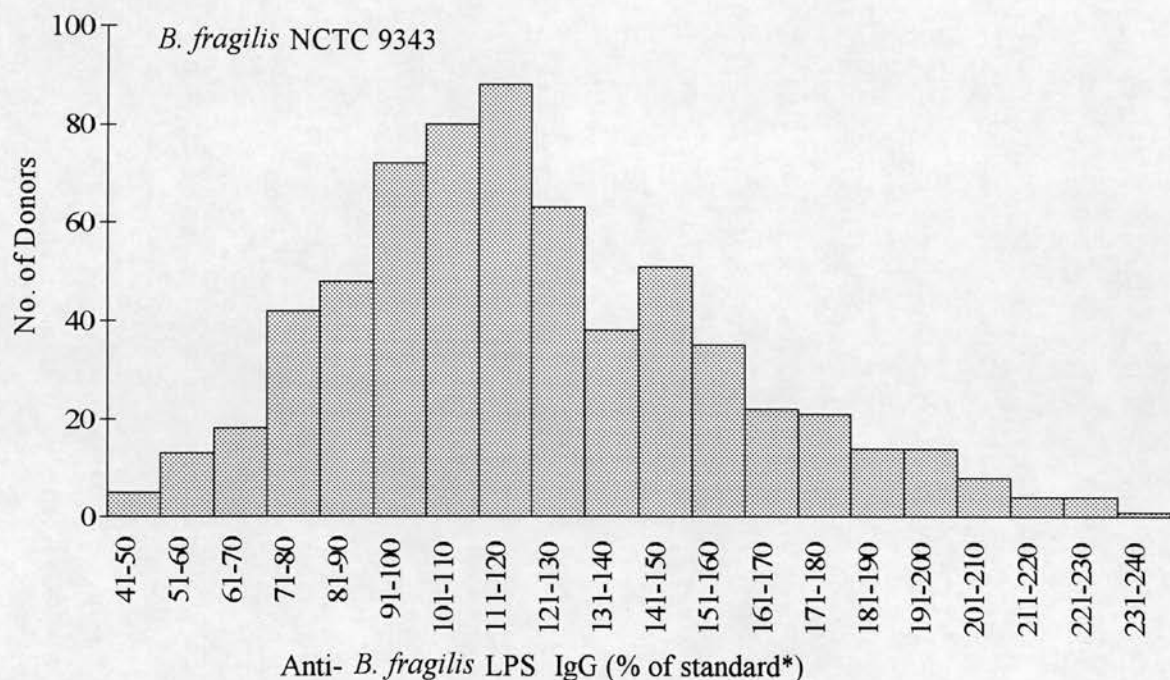


Figure 37. Distribution of anti-LPS IgG in 641 blood donors by quantitative screening ELISA. *Serum from a donor expressing close to median levels of IgG to all LPSs tested, selected from the preliminary screen of 50 random blood donors was included on all plates to standardise OD readings. Standard = 100%. Each donor serum was tested in duplicate.

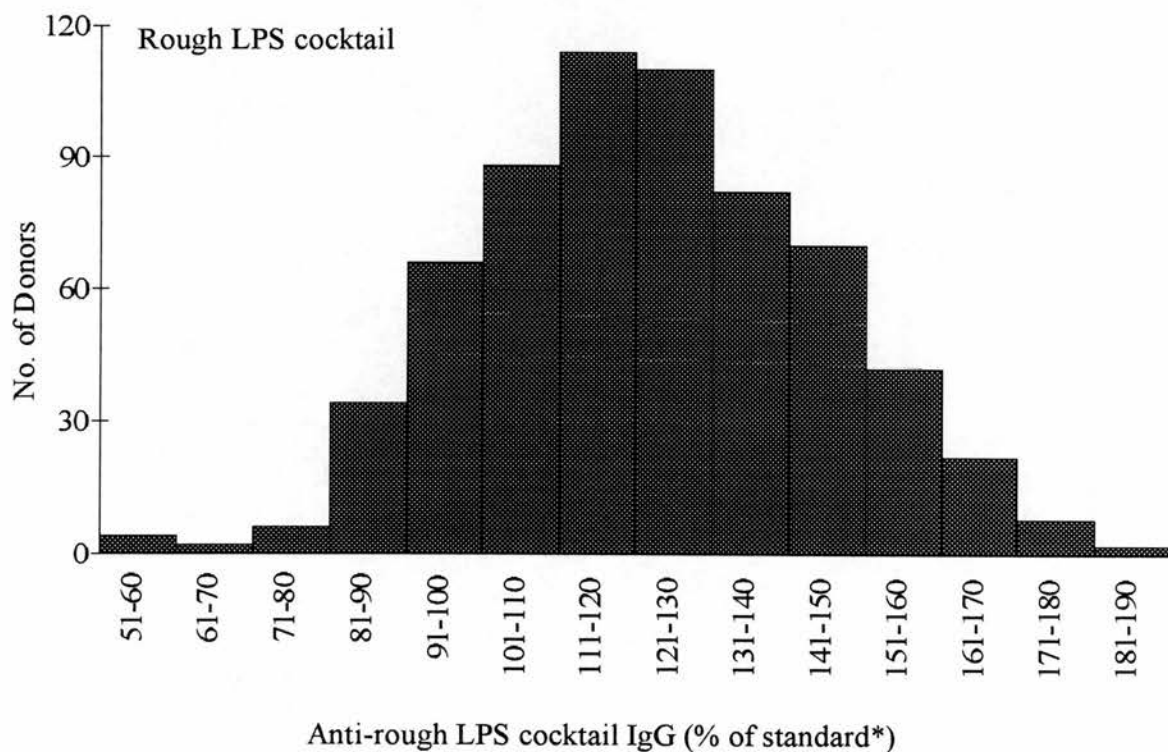
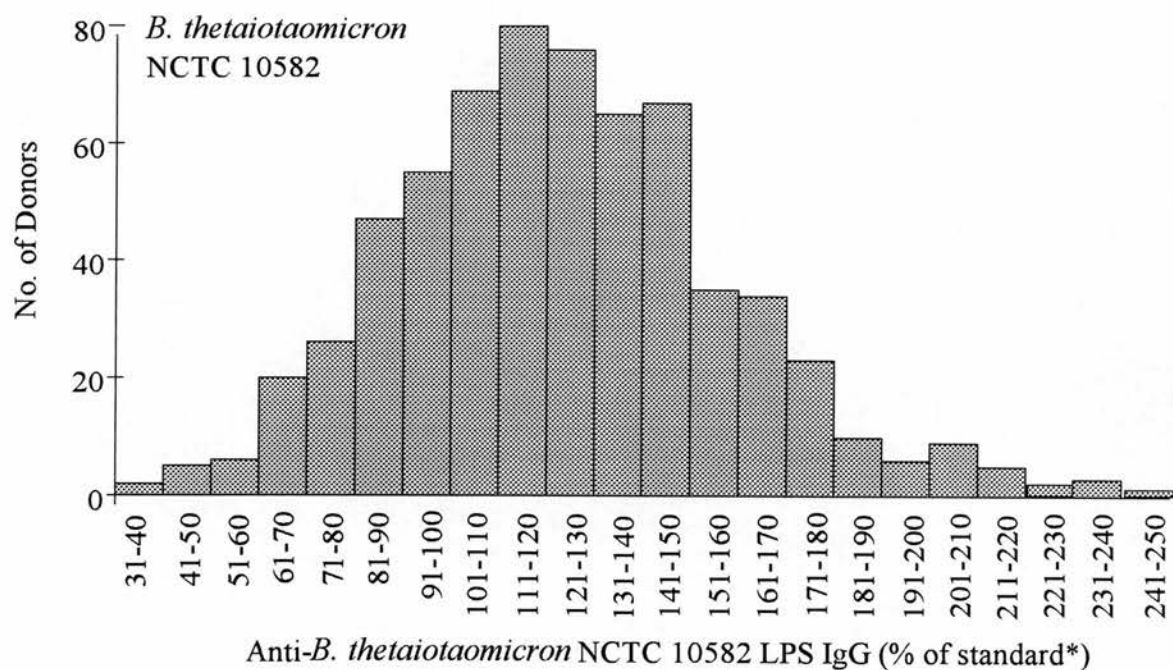


Figure 37 (continued).

days was also tested. The results are shown in Figure 38. Although no cases were identical, several broad observations could be made on the overall trends of results. In every SIRS patient, IgG levels (especially IgG against *B. fragilis* LPS) fluctuated over time to a greater extent than levels in a healthy subject. Fluctuations in IgG against all of the Ags followed similar overall trends, and in most cases anti-bacteroides LPS IgG, especially that against *B. fragilis*, showed greater fluctuations than the anti-enterobacterial / *Pseudomonas* LPS IgG. In five out of six survivors, IgG levels to all of the Ags had increased overall at the end of the sampling period. In five of the six non-survivors, levels stayed approximately the same or decreased, with an exception showing increasing levels to *B. fragilis* LPS. In five out of six non-survivors, IgG levels to *B. fragilis* LPS were higher than those against the other LPSs, and in the sixth non-survivor, IgG levels to all Ags were very low throughout the whole sampling period. Three out of six survivors and three of six non-survivors had low initial levels of anti-LPS IgG (i.e. below median levels).

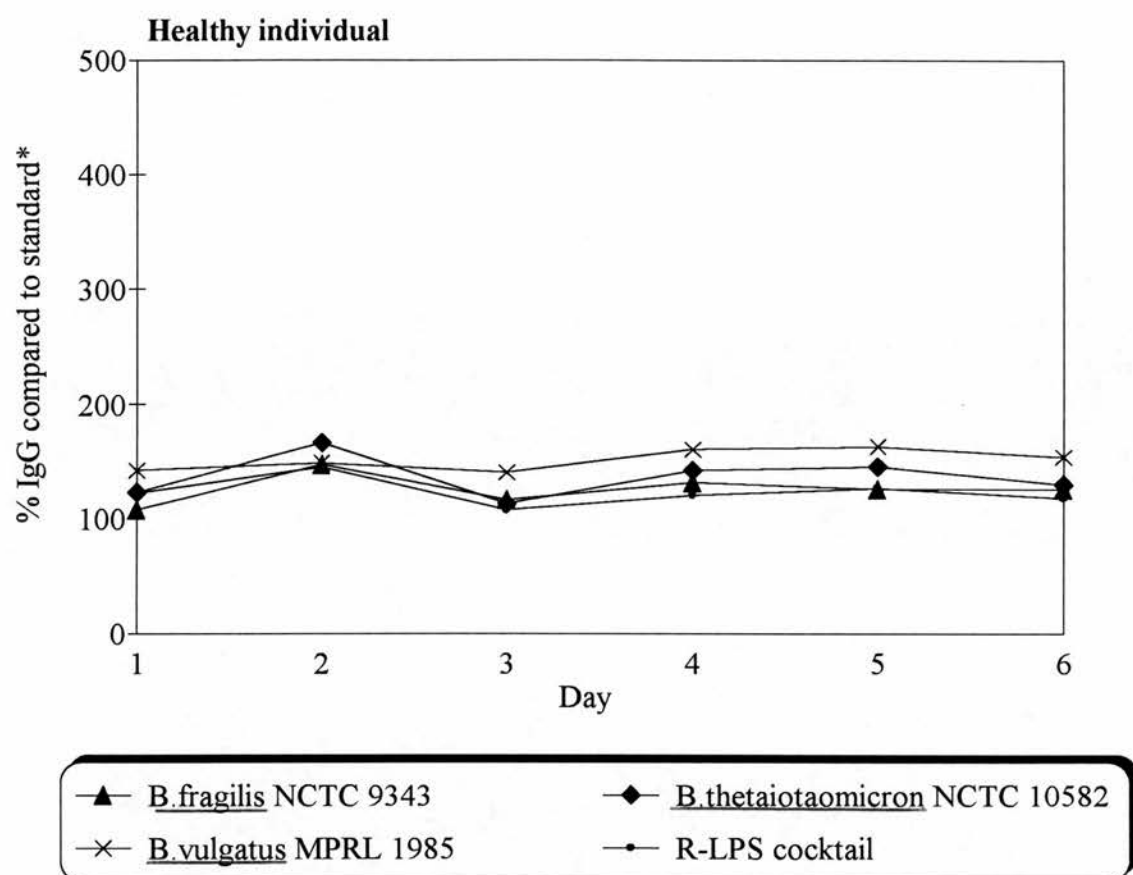


Figure 38. Kinetics of anti-LPS IgG response in 12 SIRS patients (six survivors, six non-survivors) and in a healthy individual over six- to nine-day periods. *Standard = 100%; serum from a donor expressing close to median levels of IgG to all LPSs tested, selected from the screen of 641 random blood donors, was included on all plates to standardise OD readings. Points shown are average percentages calculated from at least two replicates.

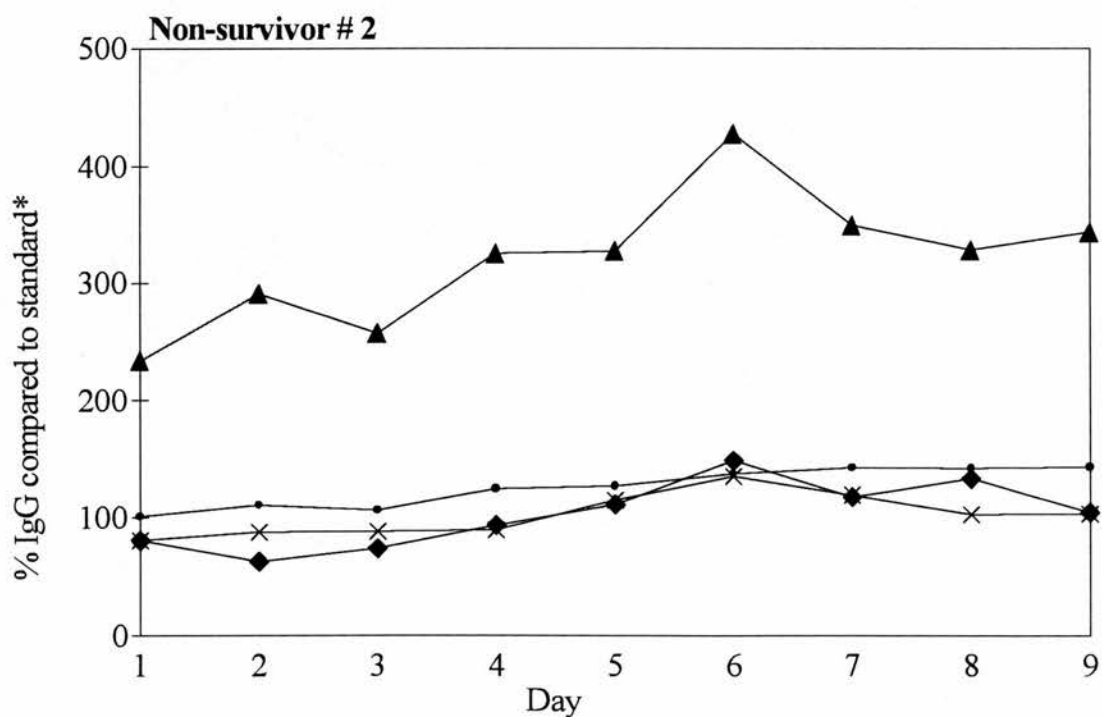
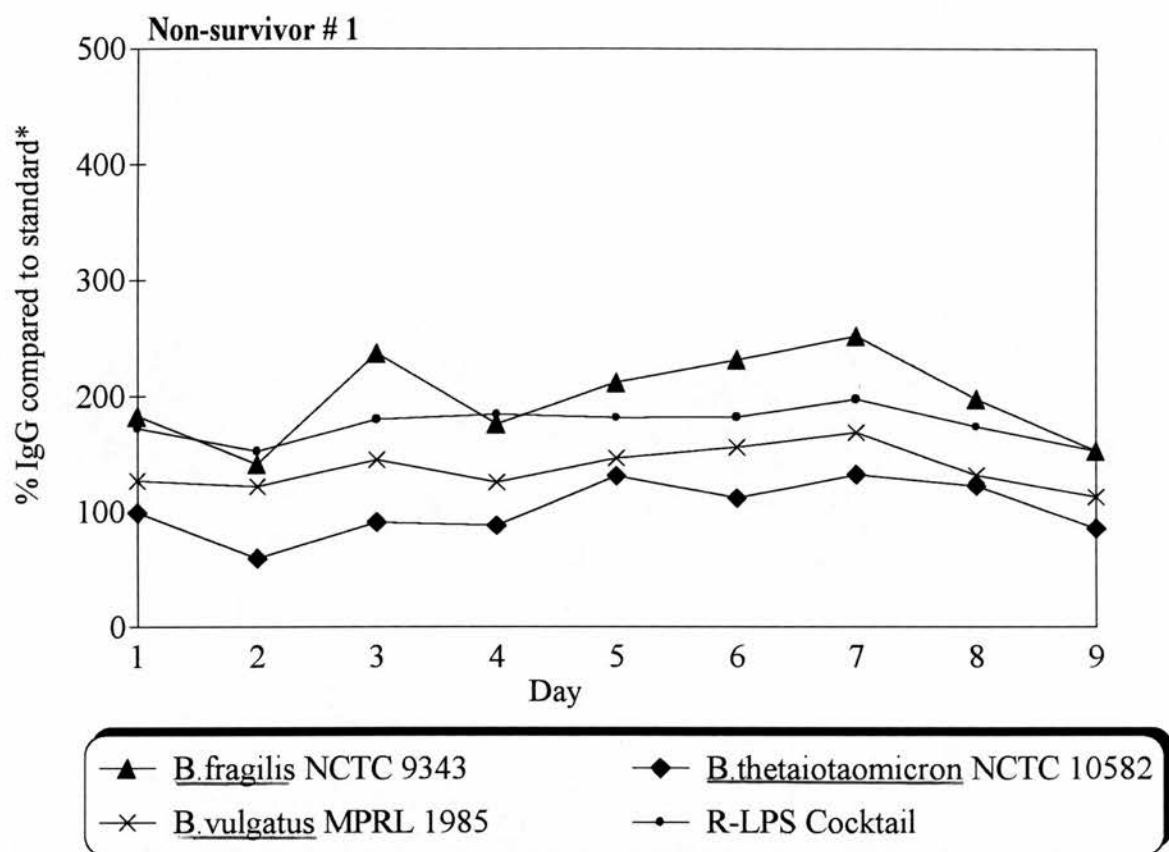


Figure 38 (continued).

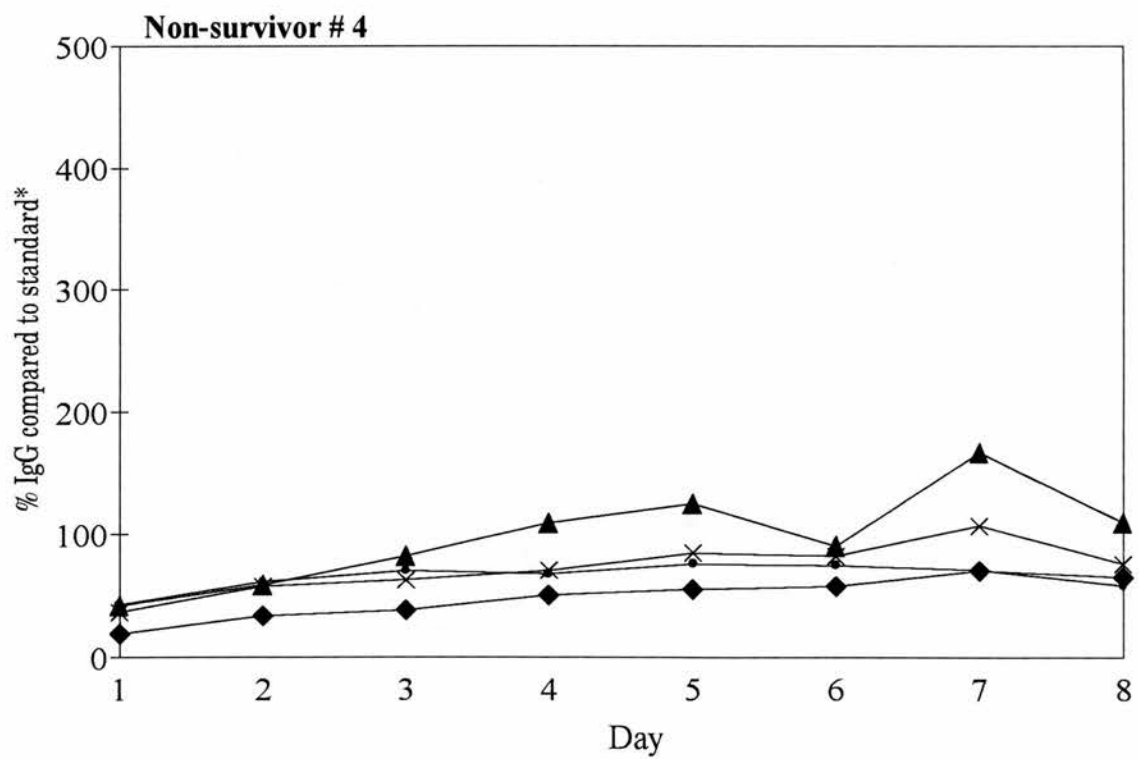
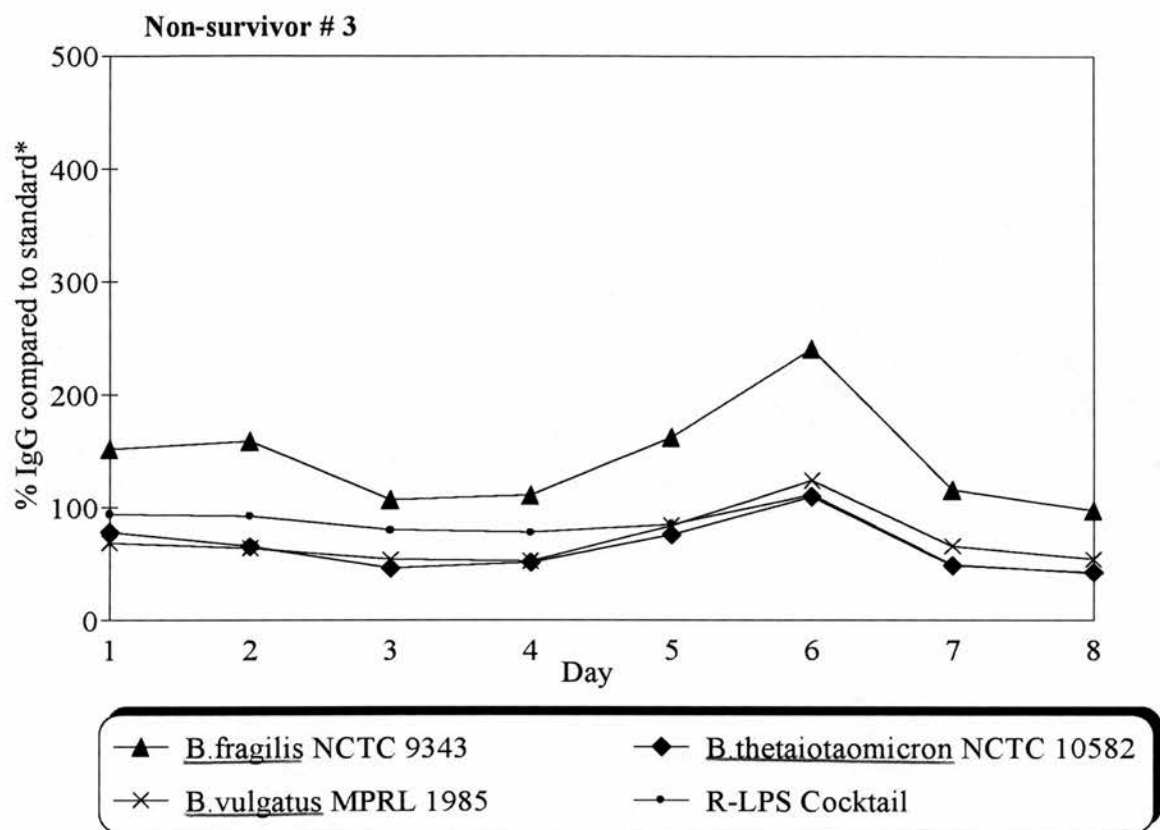


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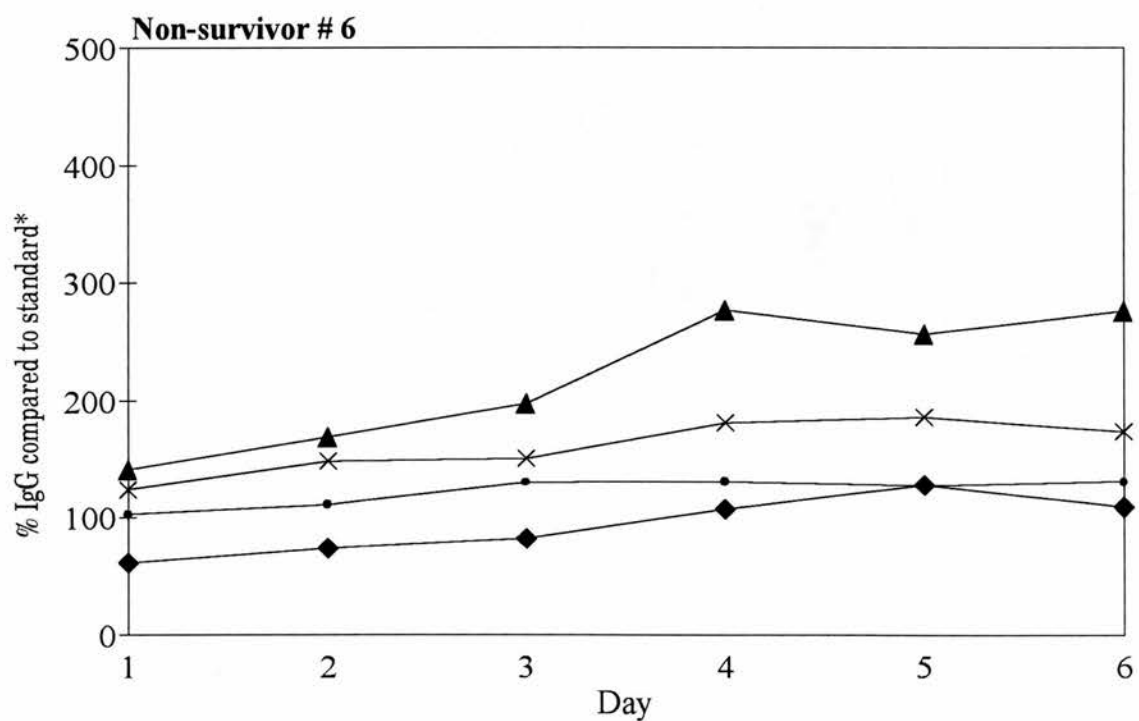
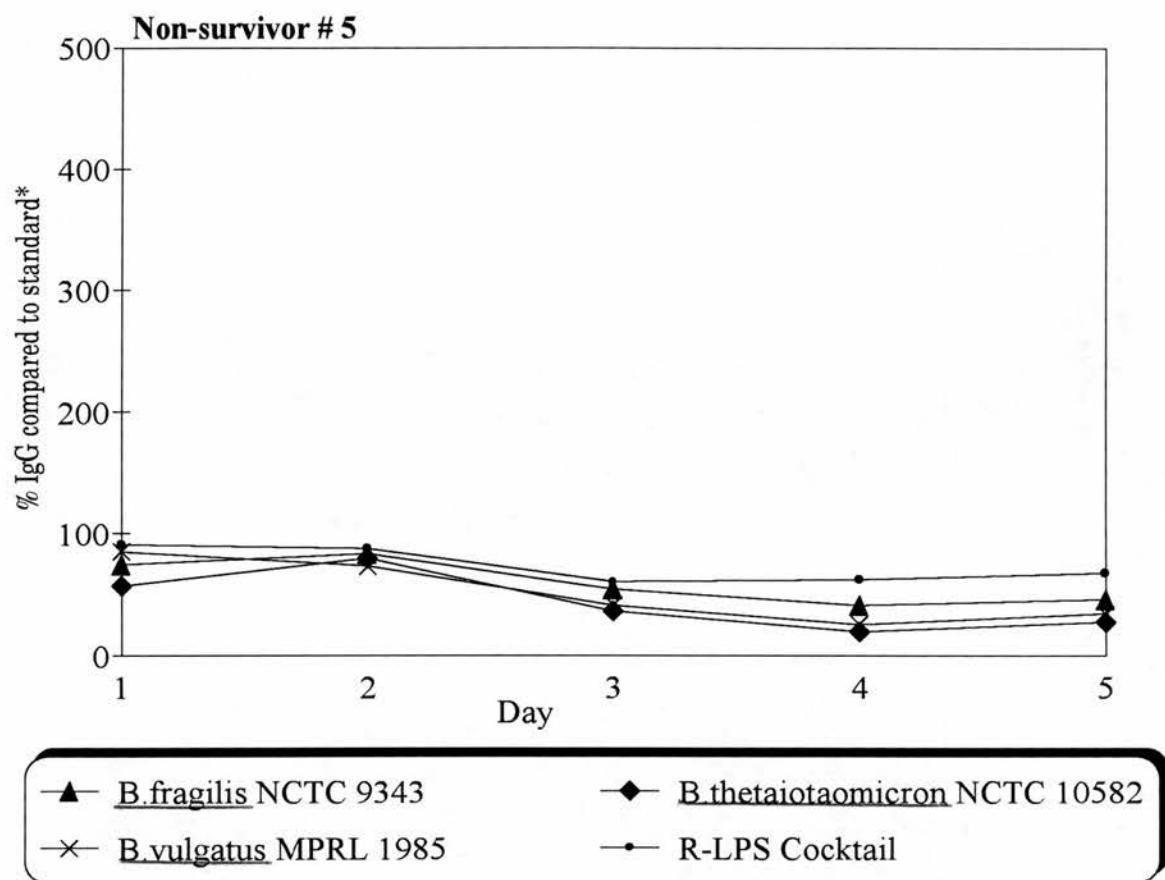


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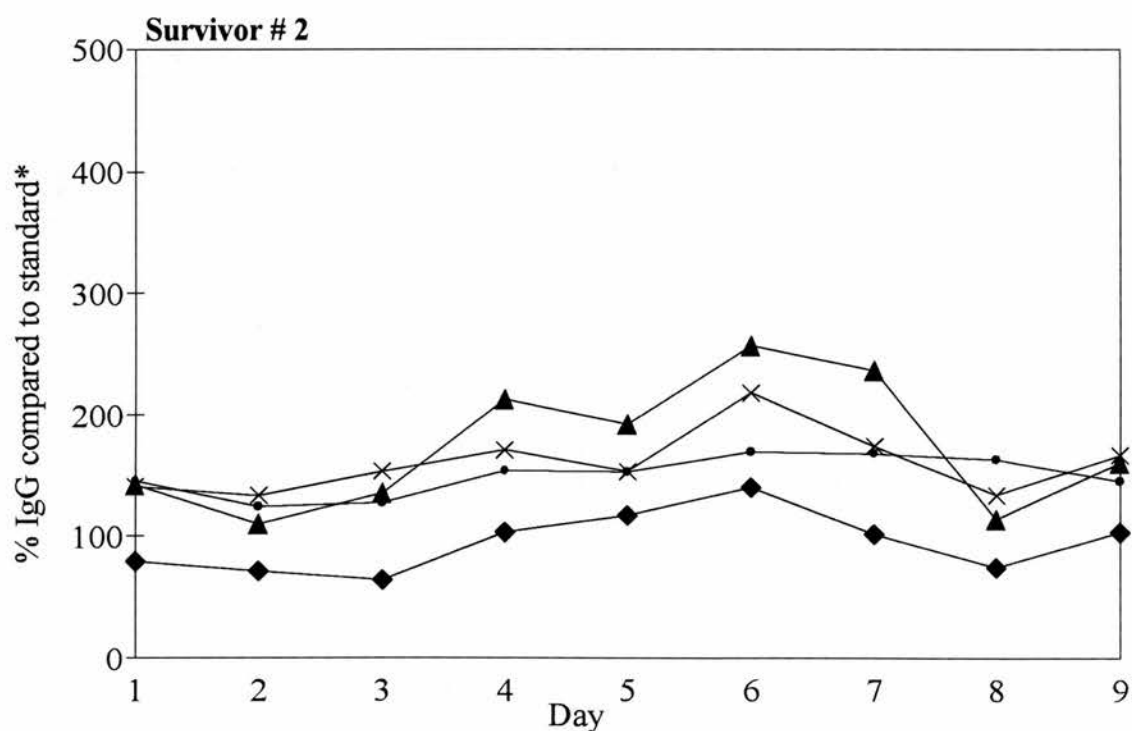
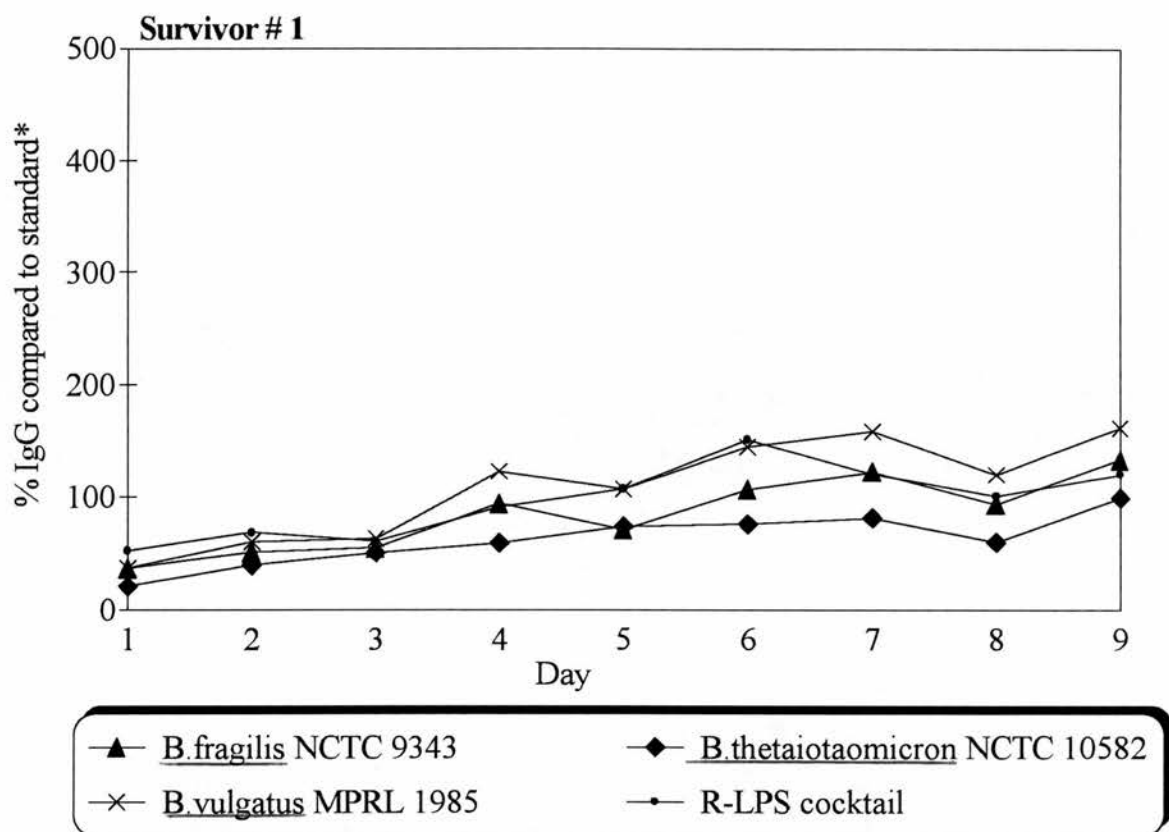


Figure 38 (continued).

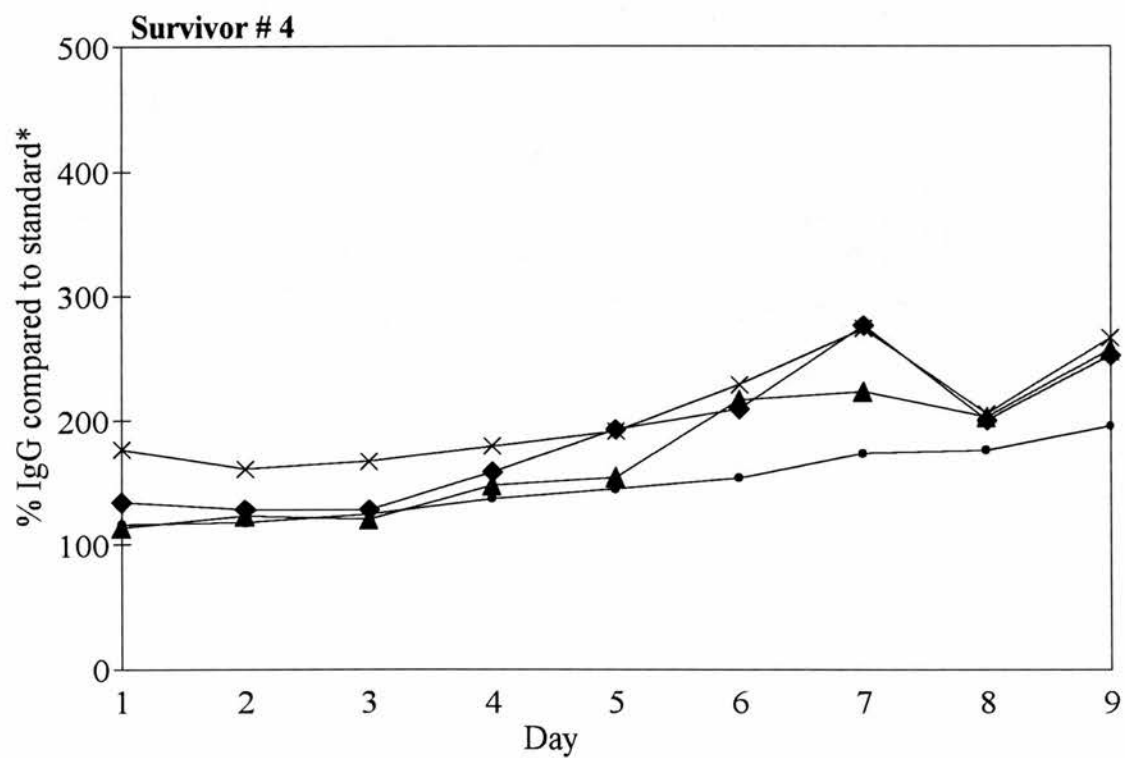
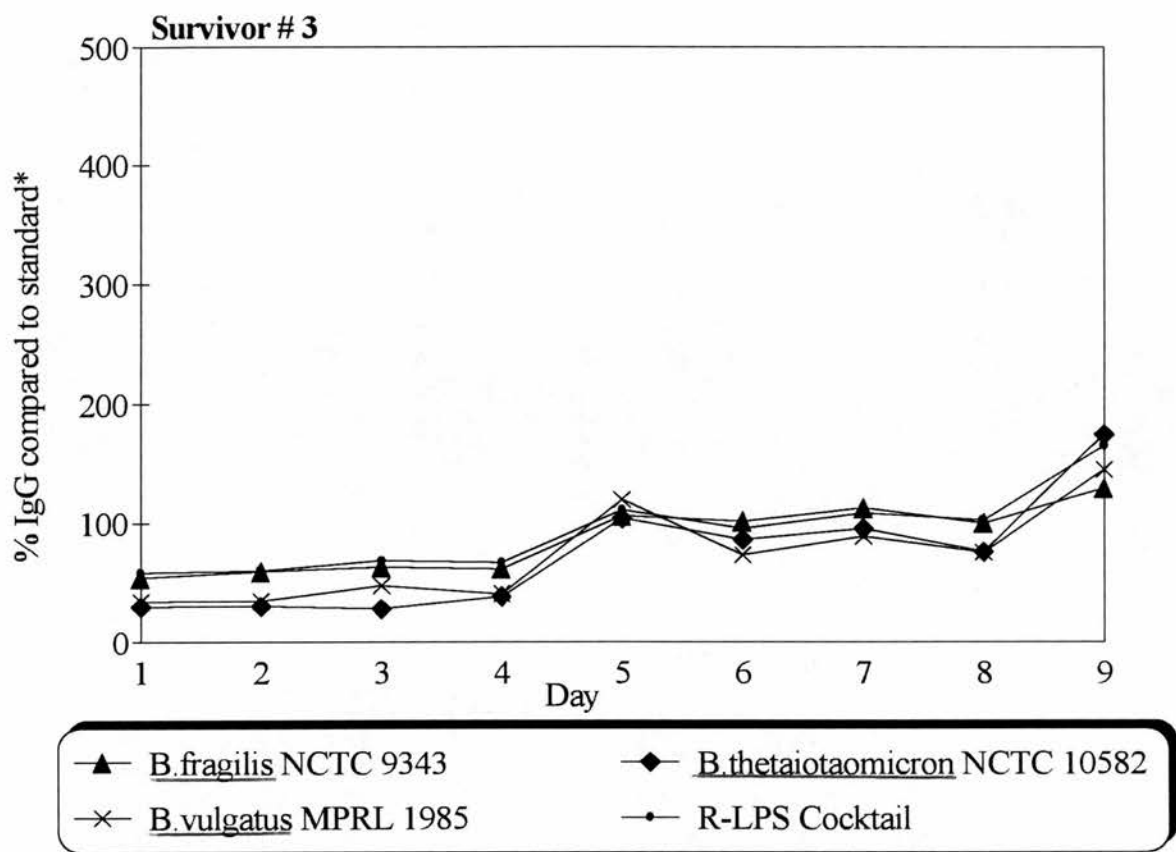


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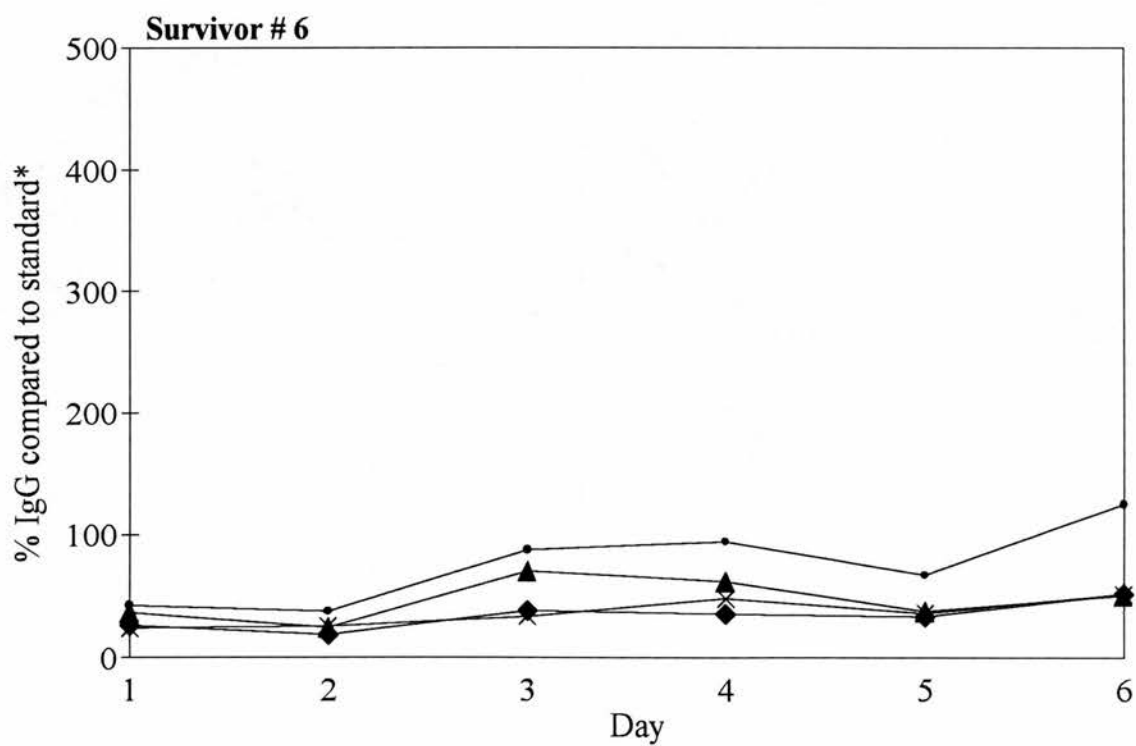
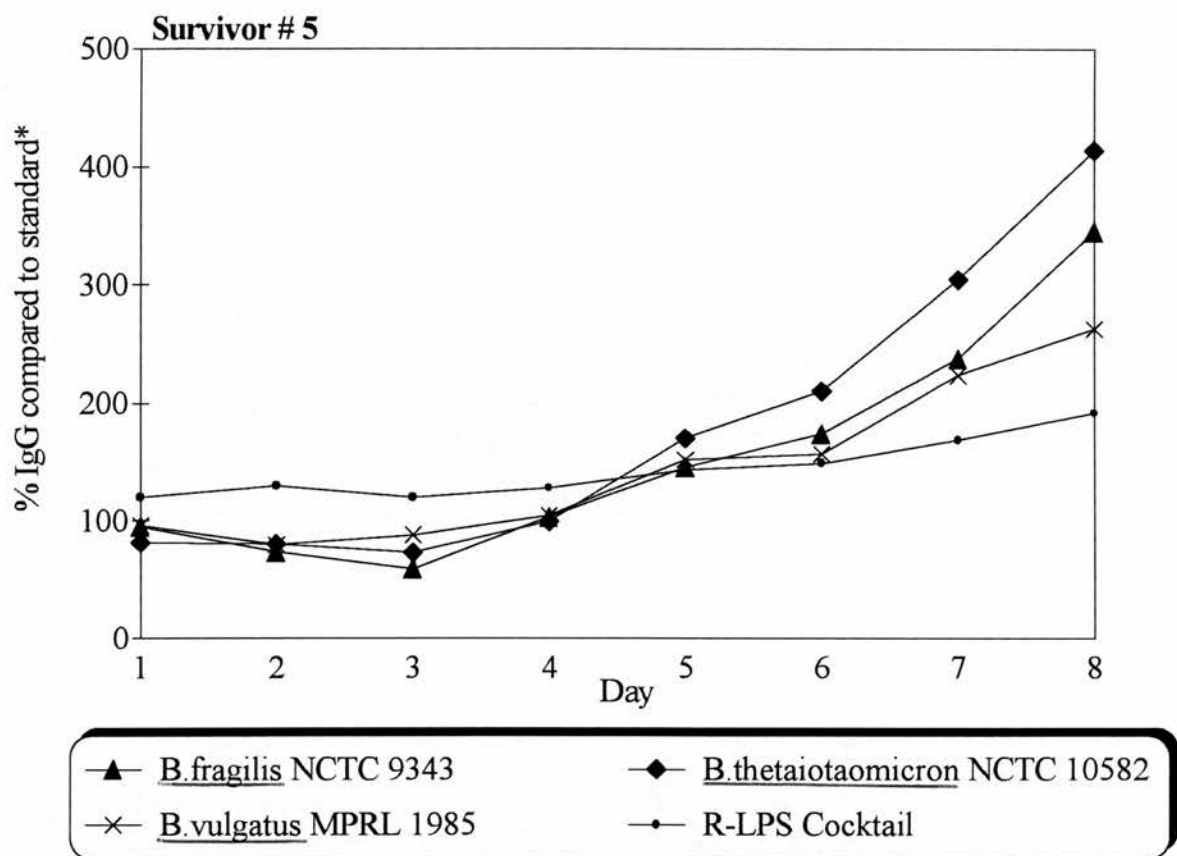


Figure 38 (continued).

3.4.8 COMPARISON OF TWO CONJUGATES TO DETECT SERUM IgG

In all previous experiments, the conjugate used to detect serum IgG recognised the whole IgG molecule (i.e. heavy and light chains). This conjugate was initially chosen to detect maximum amounts of Ab. However, there could have been some cross-reactivity between the light chains of different Ig classes. To assess whether this was the case, and therefore whether Ab classes other than IgG had been detected in the previous experiments, doubling dilutions of a serum sample from one SIRS patient (non-survivor # 4) and the healthy individual were tested in ELISA for IgG against *B. fragilis* NCTC 9343 and the R-LPS cocktail. The IgG was detected with the whole molecule-specific conjugate used above, and with a conjugate specific for the heavy (γ) chain of IgG only, and the two sets of results were compared. All of the samples were tested at the same time, so ODs were compared directly. The results are shown in Figure 39, and in each case, they show that differences between the two conjugates, particularly in the straight line regions of the graphs, were extremely small, and it therefore appeared that the anti-whole molecule IgG conjugate was detecting mainly IgG and very little else.

3.4.9 COMPARISON OF BINDING OF IgG IN ELISA TO LIPOPOLYSACCHARIDE ALONE WITH LIPOPOLYSACCHARIDE COMPLEXED WITH POLYMYXIN B SULPHATE

In all previous experiments, LPS had been complexed with polymyxin B sulphate prior to coating it onto microtitre wells, as it has been previously reported that this enhances LPS binding (Scott & Barclay 1987). As controls for previous experiments, LPS was coated to plates with and without complexing to polymyxin B sulphate and then tested in ELISA, in order to find out whether binding was enhanced. To check that human IgG did not bind to polymyxin B sulphate alone, it was coated onto plates alone and tested in ELISA. In addition, to see whether polymyxin B sulphate was preferentially binding certain parts of the LPS molecule and not others, LPS samples

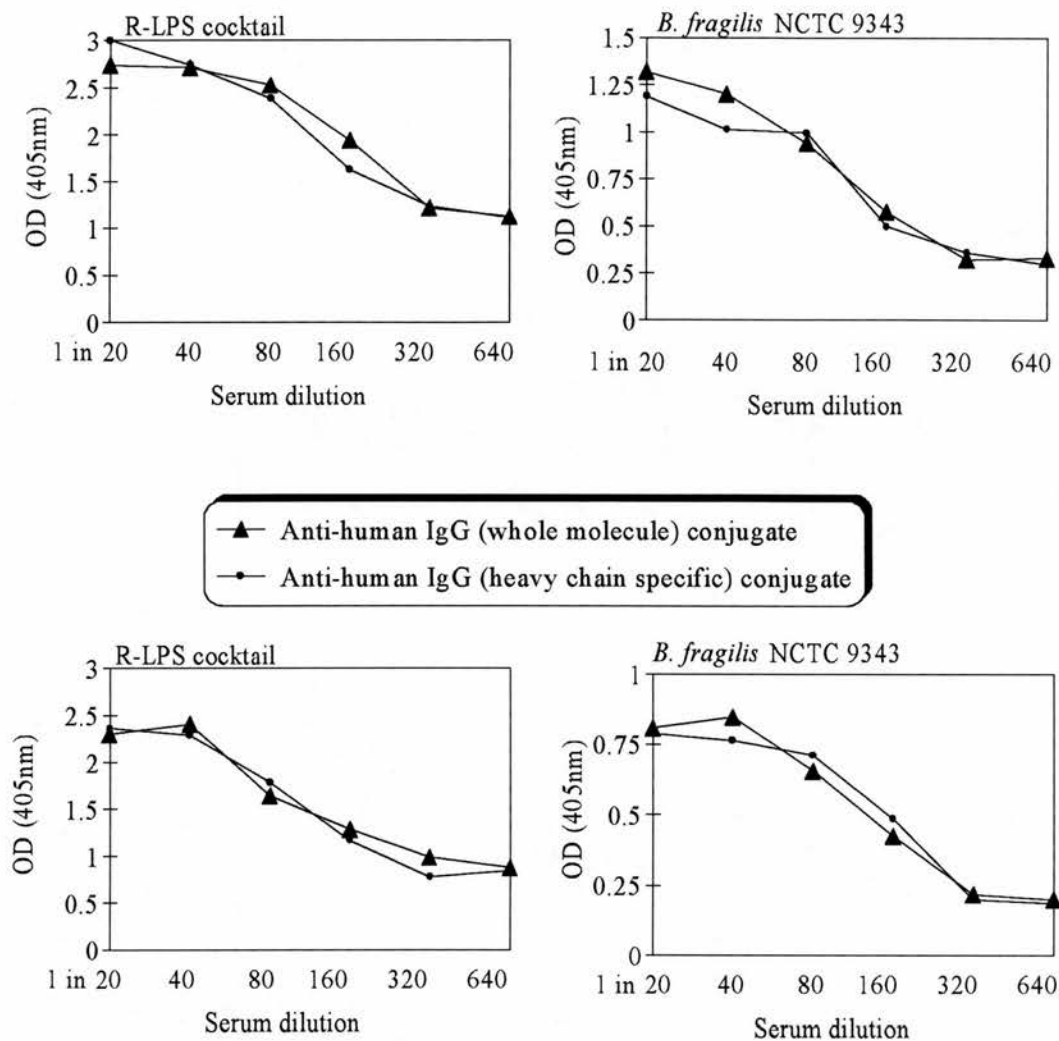


Figure 39. Comparison of the use of an anti-human IgG (whole molecule) conjugate with an anti-human IgG (γ chain specific) conjugate to measure anti LPS IgG levels in serum from **A:** a SIRS patient (non-survivor # 4) and **B:** a healthy individual. Points shown are means calculated from four replicates.

were complexed with polymyxin B sulphate, ultracentrifuged, and the pellet (polymyxin B sulphate / LPS complex) and supernate (LPS not complexed) were compared with untreated LPS samples by PAGE and silver staining. For the ELISAs, one SIRS patient's consecutive serum samples were used (survivor # 5), and were tested against the four LPS samples as before. The results are in Figure 40. In all cases, binding of IgG to polymyxin B sulphate alone was negligible, with ODs comparable to background levels (not shown). The trends of IgG binding to the complexed and non-complexed LPSs were similar, but complexing of the LPS to polymyxin B sulphate enhanced the overall amount of IgG bound, suggesting that the complexing step enhanced LPS binding to the plates. In addition, complexing appeared to increase the sensitivity of the ELISA, with differences between samples being more pronounced when the complexed LPSs were used.

The silver-stained PAGE profiles of the LPSs are shown in Figure 41. The *S. typhimurium* Rc 878 LPS was chosen as a "representative" LPS from the cocktail for this experiment, and in this case, all of the LPS appeared to have complexed with polymyxin B sulphate; ultracentrifugation of the polymyxin B sulphate / LPS complex yielded no material in the supernate. For all *Bacteroides* strains tested, there was material present in the supernate, indicating that not all of the LPS had complexed with polymyxin B sulphate. However, polymyxin B sulphate did not appear to preferentially bind certain parts of the LPS, as the LPS profiles of the supernates and the pellets were similar. The main difference was that there was more low *Mr* material in the polymyxin B sulphate / LPS complex than in the uncomplexed LPS in the supernate, but this could have been due to the fact that there was a greater concentration of LPS in the pellet compared to the supernate. Polymyxin B sulphate alone did not produce any bands on the silver-stained gel (result not shown).

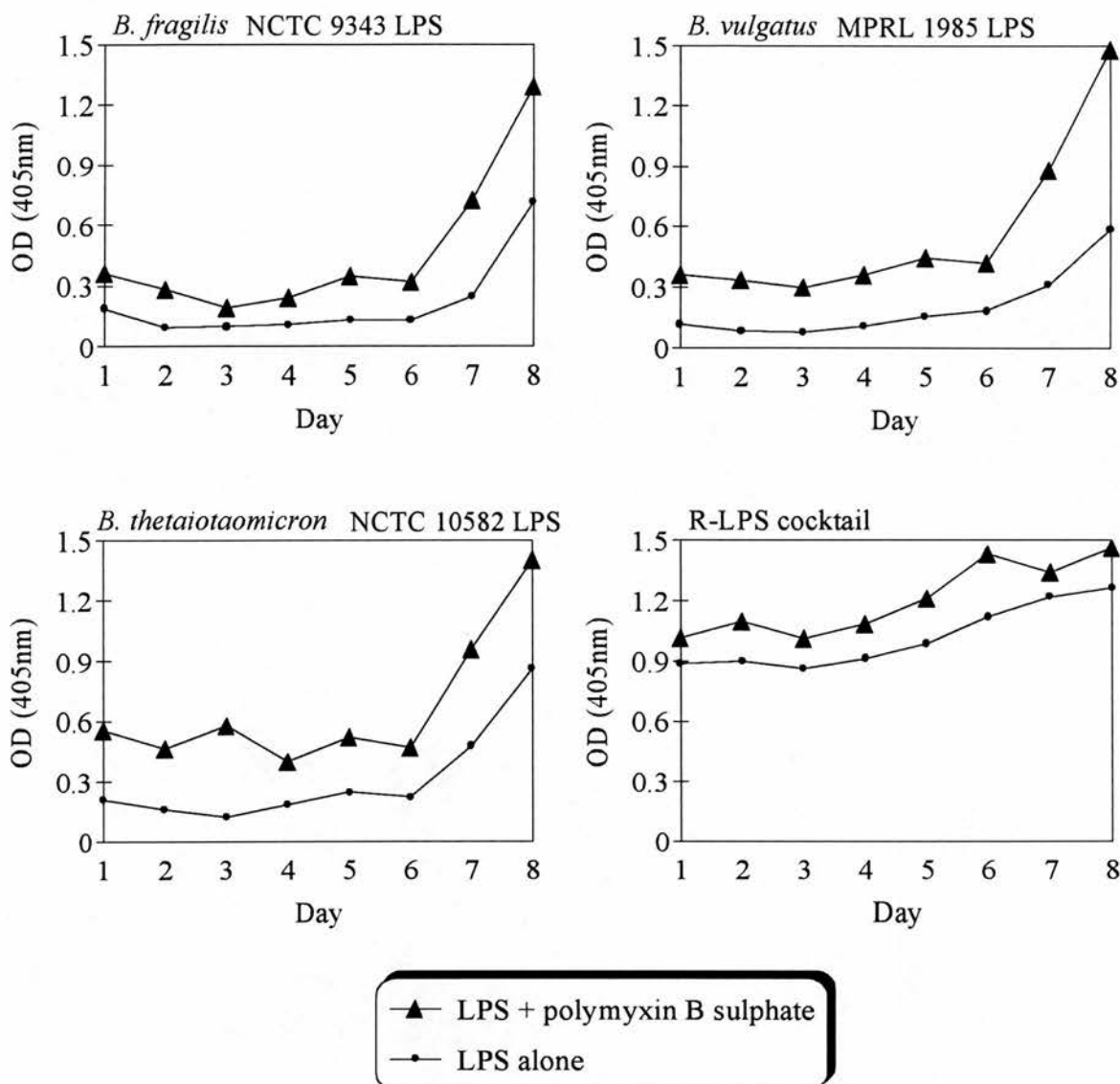


Figure 40. Anti-LPS IgG levels detected in serum samples collected over eight consecutive days from SIRS patient (survivor # 5) using either LPS complexed with polymyxin B sulphate or LPS alone coated onto microtitre plates. Results shown are means calculated from two replicates.

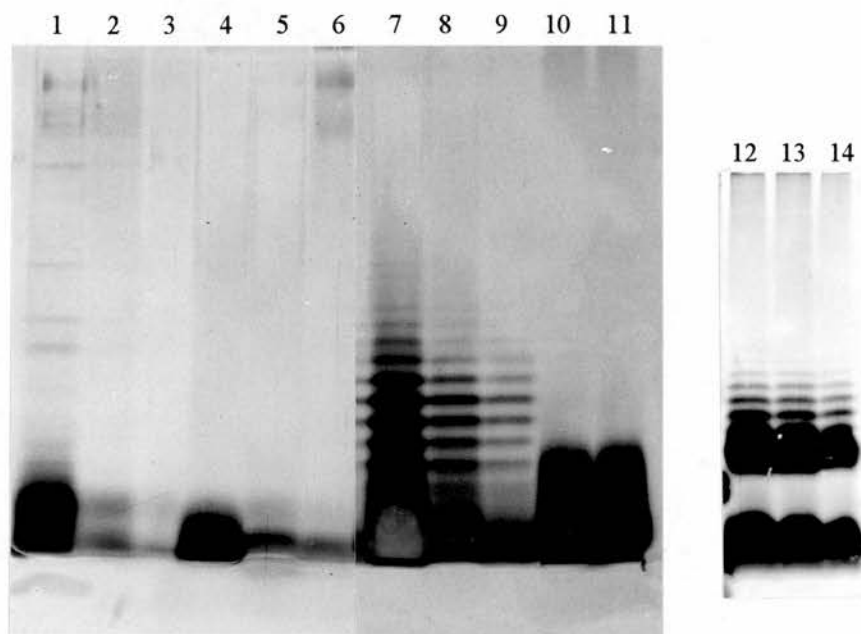


Figure 41. Silver-stained PAGE (14% acrylamide) LPS profiles of three aqueous phenol-extracted bacteroides LPSs and PCP-extracted LPS from *S. typhimurium* Rc 878 (20 μ g / track). LPSs were complexed with polymyxin B sulphate, ultracentrifuged, and the resultant material in the pellet (P) and the supernate (S) is shown with untreated controls (U). Tracks: **1**, *B. fragilis* NCTC 9343 (U); **2**, *B. fragilis* NCTC 9343 (P); **3**, *B. fragilis* NCTC 9343 (S); **4**, *B. fragilis* MPRL 1504 (U); **5**, *B. fragilis* MPRL 1504 (P); **6**, *B. fragilis* MPRL 1504 (S); **7**, *B. vulgatus* MPRL 1985 (U); **8**, *B. vulgatus* MPRL 1985 (P); **9**, *B. vulgatus* MPRL 1985 (S); **10**, *S. typhimurium* Rc 878 (U); **11**, *S. typhimurium* Rc 878 (P); **12**, *B. thetaiotaomicron* NCTC 10582 (U); **13**, *B. thetaiotaomicron* NCTC 10582 (P); **14**, *B. thetaiotaomicron* NCTC 10582 (S).

3.4.10 ANTI-LIPOPOLYSACCHARIDE IgG LEVELS IN INFLAMMATORY BOWEL DISEASE PATIENTS

Serum IgG levels against the three bacteroides LPSs and the enterobacterial / *Pseudomonas* LPS cocktail were investigated in 28 IBD patients. The results are shown in Figure 42. ODs (and therefore IgG levels) were standardised in the same way as IgG levels of the SIRS patients (Section 3.4.7), using the donor serum selected from the screen of 641 blood donors which had close to median levels of IgG against all of the Ags. With the exception of two patients (# 16 and 27), all had serum IgG levels against all of the Ags tested which were equal to, or higher than the median levels in the random blood donor population. Of the two patients with below median levels, one had oesophagitis and the other had IBD of unknown cause. At the other extreme, two patients, one with CD (# 2) and one with coeliac disease (# 28) had consistently high levels of IgG against all of the Ags. Due to the small total number of patients in this study (28), the small number with UC (2) and the relatively large number for which a diagnosis was not available (10) it was not feasible to try to compare IgG levels between people with different diseases.

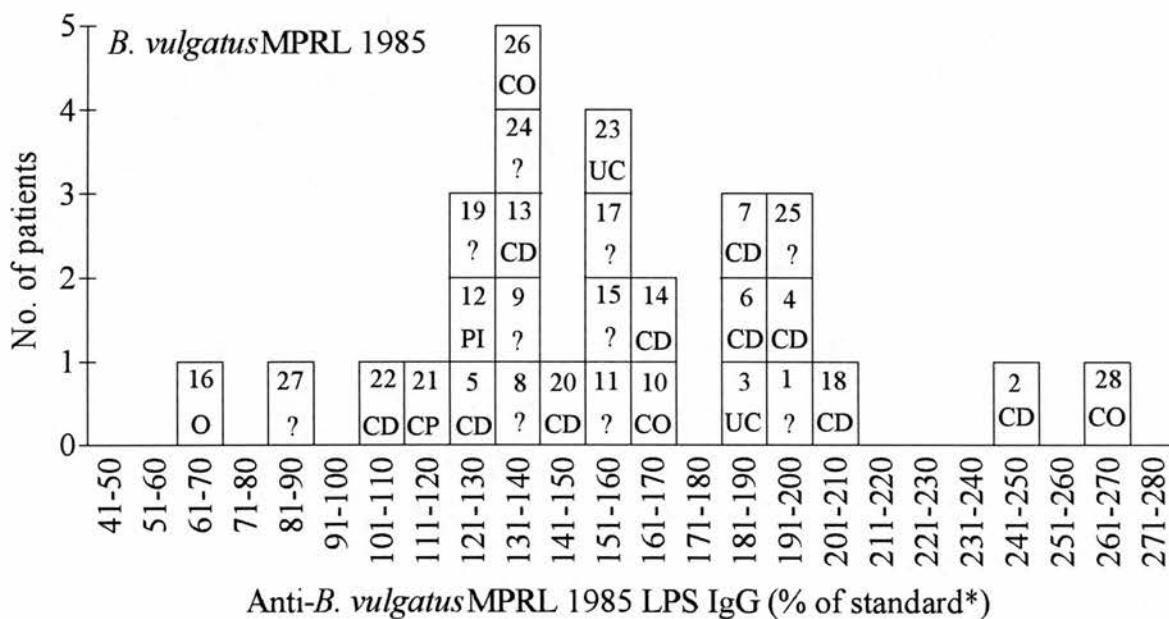
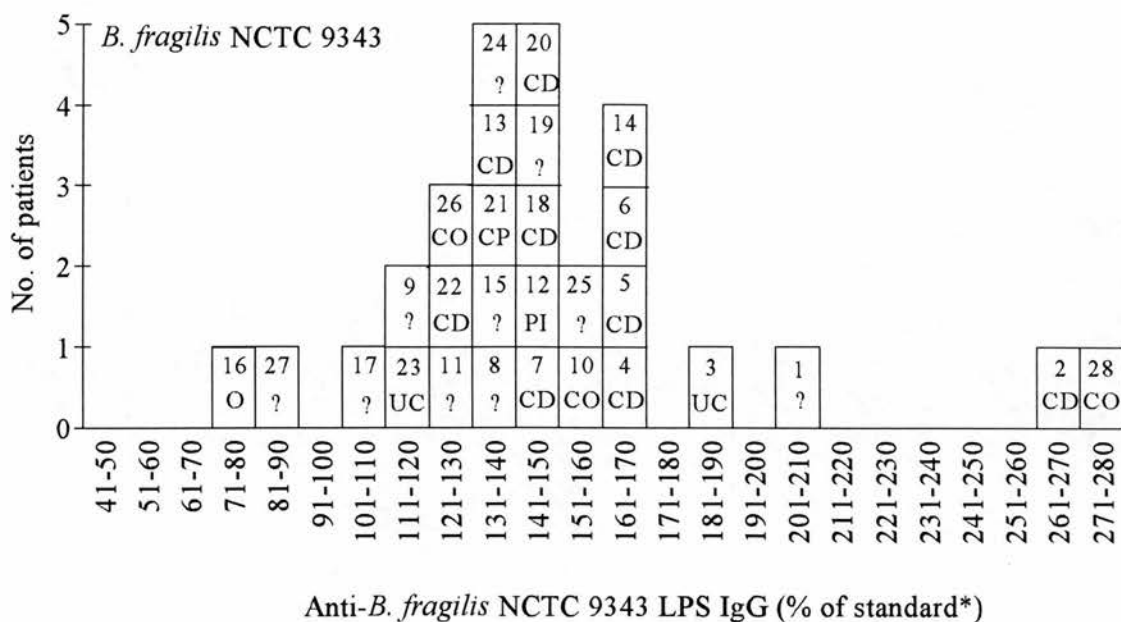
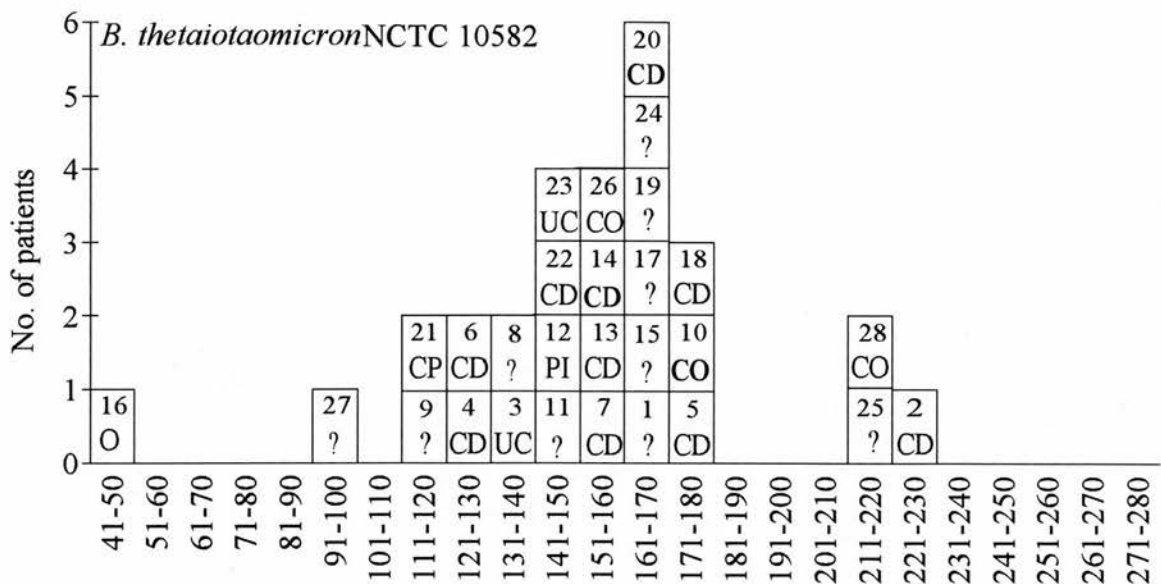


Figure 42. Distribution of anti-LPS IgG in 28 IBD patients by quantitative screening ELISA. OD readings were standardised as previously described (Section 3.4.7) using the same donor serum. *Standard = 100%. Each serum was tested four times. Abbreviations: O = oesophagitis, CP = constipation, PI = pancreatic insufficiency, CO = coeliac disease, ? = diagnosis unknown or not available. Numbers in boxes = Patient No.



Anti-*B. thetaiotaomicron* NCTC 10582 LPS IgG (% of standard*)

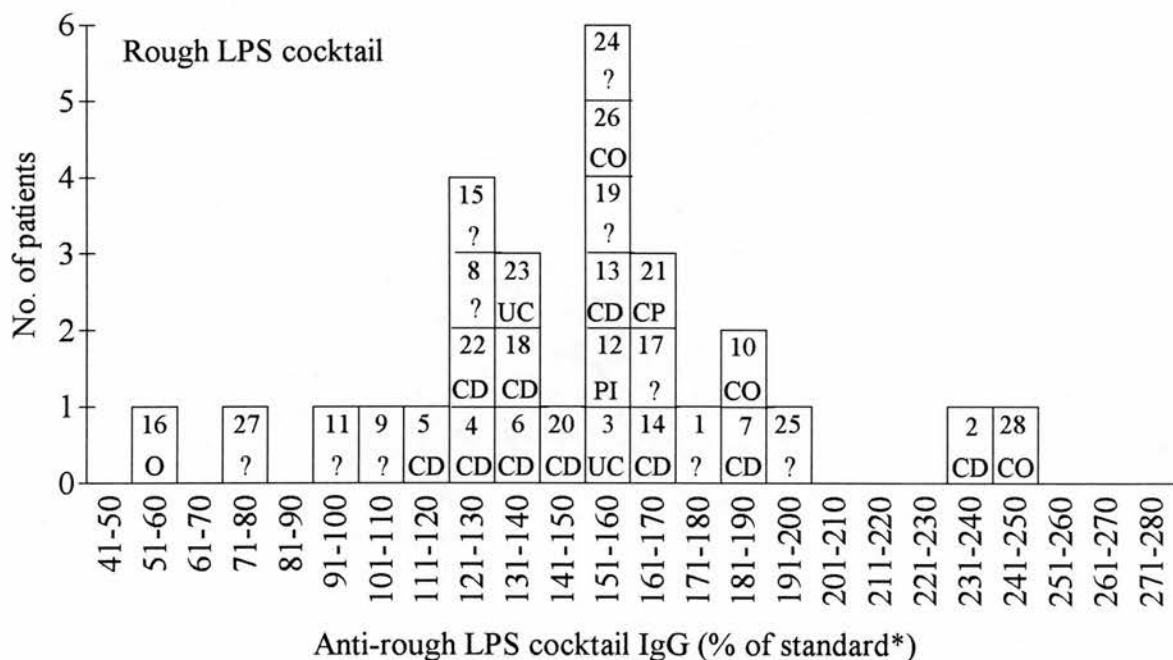


Figure 42 (continued).

CHAPTER 4

DISCUSSION

This thesis has examined the interaction between bacteroides and selected aspects of the immune system, both in health and disease. Particular attention has been paid to bacteroides cell surface Ags, firstly because these are likely to be the primary sites of interaction with the immune system and secondly because the nature of the cell surface of bacteroides is so poorly understood.

Pathogenesis is multi-factorial (Smith 1984), and results obtained in this thesis, together with previous work demonstrate that bacteroides are no exception to this rule.

4.1 EXPRESSION OF BACTEROIDES CELL SURFACE ANTIGENS IN DIFFERENT ENVIRONMENTS

It is well recognised that alterations in growth environment often result in significant changes in bacterial cell surface Ag expression (Brown & Williams 1985). Despite this, many previous studies of bacterial virulence have been carried out with bacteria grown in one medium only, and in many cases this has been a nutrient-rich laboratory medium, which provides a very different growth environment from that encountered *in vivo*.

Results obtained in this thesis have demonstrated that growth environment can significantly alter the expression of cell surface Ags in *Bacteroides* species, and therefore demonstrate that growth environment should be taken into consideration when studying pathogenicity.

4.1.1 EXPRESSION OF CAPSULAR POLYSACCHARIDE

In virtually all cases, degree of encapsulation altered with growth environment, including in the two *B. fragilis* strains examined under stress conditions (when chelators were added to the medium to deplete essential cations). It should be mentioned at this point that the capsule of *B. fragilis* NCTC 9343 grown in PPY has been examined using MAbs (S. Patrick, personal communication). It was found that the "small capsules" (from cells at the 20-40 and 40-60% Percoll interfaces) were actually small versions of large capsules - i.e. antigenically they were large capsules, although phenotypically they appeared to be small capsules. On the other hand, the capsule of this strain grown in VT at the same Percoll interfaces was antigenically and phenotypically small. To date, it is not known for definite whether similar antigenic variation occurs in other *B. fragilis* strains. A previous study by Pantosti *et al* (1992) showed limited cross-reactivity of polyclonal and monoclonal Abs raised against the CP of *B. fragilis* NCTC 9343 with the CP of other *B. fragilis* strains; only 17% of strains tested reacted with hyperimmune polyclonal rabbit antiserum raised against the CP of *B. fragilis* NCTC 9343, and only 6% reacted with MAbs against PSA and PSB of NCTC 9343. This suggests that there is considerable between-strain variation in CP structure of *B. fragilis*, in addition to the within-strain variation observed by Patrick and colleagues. It is not known whether *Bacteroides* spp. other than *B. fragilis* have sub-populations of cells with antigenically distinct capsules, although the variation in capsule sizes observed in this thesis suggests that they may do. Extensive immunological studies would be required to investigate bacteroides CP expression further.

4.1.2 EXPRESSION OF LIPOPOLYSACCHARIDE

On the whole, silver-stained LPS profiles of any given strain were not vastly different in the three media. Some differences, usually in the very low and / or the very high *Mr* regions, were apparent between the LPS profiles of certain strain sub-populations.

There was no obvious transition from R- to S-LPS in any case, although the possibility that silver staining was unable to detect changes such as this cannot be ruled out (see Section 4.2.4). Lutton *et al* (1991) demonstrated a ladder pattern characteristic of S-LPS in the EDL population, but not in the LC and SC populations of *B. fragilis* NCTC 9343 by immunoblotting; it is therefore possible that further differences would have been revealed by immunoblotting rather than silver staining in the present study.

4.2 BACTERICIDAL ACTIVITY OF HUMAN SERUM AGAINST BACTEROIDES SPECIES

The mode of action of the complement system is now largely understood, and some bacterial resistance mechanisms to its lethal effects are known. However, a major drawback in experimental work on this system is that there is no standardised method for testing serum sensitivity, and no universal definition of serum sensitivity (Taylor 1983, Crokaert *et al* 1992). Experiments performed in this thesis highlighted some of the problems which this lack of consensus can cause.

4.2.1 COMPARISON OF TWO DIFFERENT METHODS OF PROCESSING SERUM

Two different methods, termed A and B, were used to process fresh human serum; both methods were obtained from textbooks as standard procedures for serum collection and processing. However, complement activity (and therefore bacterial killing) was considerably better with serum processed by method A. Complement components are heat-labile and are unstable when left unfrozen for any length of time. Therefore, method A probably resulted in better preservation of complement activity because it was quicker than method B, and did not involve leaving the blood to clot overnight. These results highlight the fact that different methods of processing serum may significantly affect the outcome of an experiment and the conclusions drawn from it, and whichever method is used to process serum, the CH50 value should be checked to ensure that it is within the normal range.

4.2.2 THE INFLUENCE OF GROWTH MEDIUM ON SERUM SENSITIVITY OF BACTEROIDES SPECIES

Previous studies investigating the susceptibility of *Bacteroides* spp. to complement have done so after growth in one medium only. However, it is known that a change in growth environment often alters cell surface Ag expression, and also that the

expression of certain cell surface Ags can confer complement resistance on the bacterial cell. The present study demonstrated dramatic changes in the susceptibility of some *Bacteroides* strains to complement when growth medium was altered, and highlighted the need to take growth medium into consideration when studying bacterial virulence factors.

4.2.3 ORIGIN OF RESISTANT STRAINS

Of the first 12 *Bacteroides* strains tested for complement resistance (Table 4), all of the strains which became resistant after growth in HISS were of clinical origin (Table 1a). This is in agreement with a previous study by Casciato *et al* (1979), who found that faecal isolates of *Bacteroides* were significantly more sensitive to serum than clinical isolates. It is logical that virulent strains which are able to resist complement, and also possibly produce other virulence factors, are more likely to cause infection than their less virulent counterparts. All 12 of the *B. fragilis* strains subsequently tested became complement resistant following growth in HISS (Table 5). Nine of these strains were of clinical origin, and one was from faeces (Table 1a). The observation that all *B. fragilis* strains tested had the ability to become resistant to complement following growth in HISS may explain partially why *B. fragilis* is more pathogenic than other members of the genus.

4.2.4 MECHANISM OF COMPLEMENT RESISTANCE IN *BACTEROIDES FRAGILIS*

Degree of encapsulation, OMP and LPS expression, and binding of C3b to the cell surface was investigated in *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504, in order to try to discover the biochemical basis for the observed changes in complement resistance with growth medium. The presence of a capsule did not appear to be responsible for complement resistance; *B. fragilis* MPRL 1504 grown in HISS was mainly non-capsulate, and was resistant to complement, whereas the capsulate cells

(grown in VT and PPY) were sensitive. This is in agreement with a previous study, where Reid & Patrick (1984) found the non-capsulate variant of *B. fragilis* ATCC 23475 to be resistant to complement, and the capsulate variant to be sensitive. On the other hand, *B. fragilis* NCTC 9343 was mainly capsulate in HISS. Of the other four strains which became complement-resistant following growth in HISS (Table 4), two were mainly capsulate and two were mainly non-capsulate. In the cases where complement-resistant cells were non-capsulate, CP was still produced but appeared to be present as cell-free slime. It is therefore possible that the cell-free CP is responsible for "mopping up" complement components at a distance from the bacterial cell. A very useful way to investigate this would be to generate mutants which were unable to produce CP of any description, and test their ability to resist complement.

Differences in OMP expression were observed for both strains grown in PPY and VT & S. Certain OMPs may be involved in conferring complement sensitivity or resistance in *B. fragilis*, but further investigations would be required to discover whether this was the case. It could prove useful to incorporate a radioactive label into the growth medium, so that active induction of OMPs could be monitored. Although no bands were present when VT & S alone, and PPY alone were electrophoresed and stained with silver, there is still a possibility that certain proteins from the growth media were associated with specific OMPs, which would cause these protein bands to appear stronger than they should have done. If this was the case, it would explain differences in this study compared to that of Patrick & Lutton (1990), who found no significant differences in OMP expression of *B. fragilis* grown in different environments.

The silver-stained aqueous phenol extracts did not reveal any major differences in the LPS profiles of either strain grown in different media. However, immunoblotting of

the aqueous phenol extracts revealed differences not observed by silver staining; dark-staining material was present in the S-LPS region of MPRL 1504, and a ladder pattern characteristic of S-LPS was evident in NCTC 9343 grown in VT and VT & S. There are several reasons why immunoblotting may have revealed differences not observed by silver staining: silver staining is less sensitive than immunoblotting, immunoblotting can reveal immunogenic material not visualised by other methods, and the carbohydrate of the ladder pattern may be resistant to oxidation by periodate - the first step in the silver stain. A possible reason why MPRL 1504 did not show a ladder pattern is that it is a very mucoid strain, and it is therefore possible that the copious amounts of slime present prevented material from entering the separating gel, or masked its presence on the gel. It would be interesting to screen aqueous phenol extracts from other *B. fragilis* strains by immunoblotting to see whether they also produced ladder patterns.

In this study, C3b bound equally well to all cells. However, it should be stressed that there could be differences in binding of C3b to bacterial cells coated onto ELISA plates compared to cells in the body.

In summary, differences in degree of encapsulation, OMPs and LPS were observed in both strains when grown in different media. In one of the strains (NCTC 9343), complement resistance could have been due, at least in part, to the presence of S-LPS. However, the complexity of the surface polysaccharide complex of *B. fragilis*, and the difficulties in purification of its different components (discussed in detail in Section 4.3) prevent any firm conclusions being drawn. The two *B. vulgatus* strains tested in this study both remained sensitive to complement, suggesting that the presence of S-LPS alone was not sufficient to confer complement resistance. However, it should be noted that the S-LPS of *B. vulgatus* has a much lower *Mr* than the classical S-LPS of enterobacteria, and a lower *Mr* than the material seen in the S-LPS region in *B.*

fragilis NCTC 9343. It is therefore possible that complement resistance is dependent on the Mr of the S-LPS. Moreover, it is possible that the presence of S-LPS in *B. fragilis* is only one of several factors which confer complement resistance, and other factors which were not examined in this study, such as secreted or cell-bound proteases, or incorrect assembly of the membrane attack complex could also contribute to complement resistance in this species. The interaction of several different factors resulting in the ability to resist complement would be a classic example of the multi-factorial nature of pathogenicity.

4.2.5 MECHANISM OF COMPLEMENT RESISTANCE IN *BACTEROIDES THETAIOAOMICRON*

The two *B. thetaiotaomicron* strains, one of which (NCTC 10582) remained sensitive to complement and the other of which (MPRL 1959) became resistant showed differences in encapsulation, OMPs and LPS profiles. However, the mechanism of complement resistance in *B. thetaiotaomicron* cannot be determined from these results alone. Later examination of a full-scale aqueous phenol extract of NCTC 10582 revealed a ladder pattern characteristic of S-LPS which was not evident in rapid phenol or Proteinase K extracts (Section 3.4.1). This suggests, as was the case with *B. vulgatus*, that S-LPS alone is not sufficient to confer complement resistance.

4.2.6 COMPLEMENT RESISTANCE OF *BACTEROIDES FRAGILIS* GROWN IN VT & S THEN PPY

The two *B. fragilis* strains tested in this experiment were initially more resistant to complement after subculture from VT & S to PPY, as opposed to sub-culture from PPY to PPY. This increased resistance was diminished on further sub-culture in PPY. This result suggests that the virulence determinant(s) which are expressed in VT & S are gradually lost. This could be because of the amount of energy required to make them. Growth in VT & S was slower than growth in PPY, possibly because

certain proteins and / or carbohydrates which are necessary for growth and survival in VT & S, and which also confer complement resistance, require a lot of energy to produce. If these molecules were not required for growth and survival in PPY, then energy generated would be used for faster growth.

4.2.7 CLASSICAL PATHWAY VERSUS ALTERNATIVE PATHWAY ACTIVATION

Initially it was attempted to look at classical and alternative pathway activation by using whole cells to absorb out specific Abs. Results suggested that in *B. fragilis* MPRL 1504 grown in PPY, some activation was occurring via the classical, and some via the alternative pathway. However, this method of trying to inhibit the classical pathway has a number of major shortcomings:

- Although specific Ab is absorbed out, there is no means of knowing whether it has all been removed, and any residual Ab left could still activate the classical pathway.
- Some Ab classes (IgA, IgG4) activate the alternative pathway.
- The amount of Ab removed will increase with increasing numbers of absorptions, but the longer the serum is left before using it in the serum sensitivity assay, the more likely it is to become complement-depleted.
- Anti-bacteroides sheep Abs which stuck to the cells during growth in VT & S and were not removed by washing would activate complement.
- The classical pathway can be initiated by means other than Ab / Ag complexes (e.g. lipid A, porins).

To circumvent these potential problems, a second method was used to look at complement activation. The addition of EGTA to serum chelates Ca^{2+} ions, and therefore specifically inactivates the classical pathway. Results from this experiment showed that the method of complement activation was dependent on both growth medium and bacterial strain. In all cases (except *B. variabilis* VPI 11368 grown in PPY where complement activation was by the classical pathway alone), activation was occurring partially via the classical, and partially via the alternative pathway. Activation of complement by *B. vulgatus* was mainly via the alternative pathway in all three media. This could be because the O-Ag is known to activate the alternative pathway (Eisen & Geftter 1990), and *B. vulgatus* has the most obvious, presumably well-exposed O-Ag of all of the *Bacteroides* spp. From these experiments alone, it is not possible to say whether the method of activation is important in complement resistance of *Bacteroides* spp., although the fact that it is activated partially by both pathways in nearly all cases suggests that if complement activation was prevented by the bacterium, it would have to prevent both pathways of activation. In these experiments, it was not possible to see whether complement was being activated in those cases where the cells were totally resistant. A useful follow-up would be to measure concentrations of complement components in serum in those cases where totally resistant cells had been added.

4.2.8 BACTERICIDAL ACTIVITY OF SERUM FROM SYSTEMIC INFLAMMATORY RESPONSE SYNDROME PATIENTS

The complement cascade is initiated as part of the pathophysiology of SIRS, resulting in depletion of all complement proteins. CH50 levels in both patients' sera were well below normal levels, with the result that the efficiency of bacterial killing was reduced. This has important implications in the management of SIRS patients - they will be less able to fight potential pathogens and more vulnerable to septicaemia and other infections than healthy individuals. Bacteria which are readily killed in serum

from a healthy individual will be more likely to survive in serum from a SIRS patient, and this should be borne in mind when studying complement resistance and any other aspect of the host / pathogen interaction in these patients.

4.3 ANALYSIS OF THE SURFACE POLYSACCHARIDE COMPLEX OF BACTEROIDES FRAGILIS

These experiments were designed to analyse the surface polysaccharide complex of *B. fragilis*, both visually and chemically, in order to try to make sense of some of the confusion which surrounds this subject.

4.3.1 ANALYSIS OF ULTRACENTRIFUGED AND NON-ULTRACENTRIFUGED AQUEOUS PHENOL EXTRACTS OF BACTEROIDES FRAGILIS

Earlier work carried out in this thesis had aroused suspicions that rapid phenol and Proteinase K extracts of *B. fragilis* were not pure LPS. Moreover, it was not clear what effect the final rotary evaporation and ultracentrifugation steps in the large-scale aqueous phenol extraction method would have on the composition of the material in the extracts. Therefore, initially two *B. fragilis* strains (NCTC 9343 and MPRL 1504) were grown in three media, aqueous phenol extractions were carried out and half of the resultant material from the aqueous phase was subjected to rotary evaporation and ultracentrifugation, whilst the other half was lyophilised without these steps.

The percentage yield from the non-ultracentrifuged extracts was greater than that from the ultracentrifuged ones in all cases except MPRL 1504 grown in VT & S. This one anomalous result was probably due to inaccuracy in dividing the original extract in two. Ignoring this result, the others suggest that ultracentrifugation was not precipitating all material in the extracts.

Further analysis of the extracts revealed differences in composition between strains, between different growth media and between ultracentrifuged and non-ultracentrifuged samples. The main observations are summarised below:

- The aqueous phenol extracts were not pure LPS, but also contained protein.
- Ultracentrifugation decreased the relative amount of protein and increased the relative amount of carbohydrate in the extracts, suggesting that the material left behind during ultracentrifugation was mainly protein. Silver staining revealed greater amounts of, or better defined low and high *Mr* material (mainly carbohydrate: see Section 4.3.2) in the ultracentrifuged, as opposed to the non-ultracentrifuged extracts.
- In both strains, growth in VT resulted in the greatest amount of carbohydrate production, followed by growth in VT & S, followed by growth in PPY. This probably reflects the amount of readily accessible carbohydrate in the growth medium; it has been found that an excess of carbohydrate, together with a lack of phosphate, nitrogen or sulphate favours CP production (Sutherland 1972). VT contains 1% glucose and is therefore a very rich source of carbohydrate. In stationary phase in VT medium, most nutrients would be in short supply, but there would still be excess glucose present which could be used for CP production.
- It had been noted previously (Section 4.2.4) that MPRL 1504 was a very mucoid strain. Chemical analysis of the surface polysaccharides confirmed this: in all cases, the amount of carbohydrate produced by this strain was nearly double that produced by NCTC 9343. This provides further evidence of variation of CP between different *B. fragilis* strains.
- Large amounts of carbohydrate in mainly non-capsulate cells (NCTC 9343 in VT and MPRL 1504 in VT & S), together with high *Mr* material present on silver-stained gels, strongly suggested that large amounts of cell-free slime were present in these cases.

- KDO measurements were negligible. This confirms previous work (Beckmann *et al* 1989) which found that it was necessary to dephosphorylate the KDO of *Bacteroides* spp. with hydrofluoric acid before it would give a positive reading in a KDO assay.

A previous study reported that extraction of *P. aeruginosa* LPS with aqueous phenol resulted in a larger amount of material in the phenol phase of the extract than in the aqueous phase (McVie 1993). To see whether this was the case in *B. fragilis*, the phenol phases were examined. In all cases, the material in the phenol phase had similar PAGE profiles to the material from the aqueous phase, but the percentage yields from the phenol phases were very low, indicating that in *B. fragilis*, the majority of material was located in the aqueous phase.

Immunoblots of the extracts, carried out using polyclonal rabbit serum raised against *B. fragilis* whole cells, failed to show low *Mr* material. This was also the case with previous immunoblots (Section 3.2). The reason for this is probably that low *Mr* material such as lipid A and LPS core is very close to, or embedded in the outer membrane of whole cells, and is therefore hidden from the immune system.

In summary, ultracentrifugation appeared to be partially "purifying" the extracts in that it was reducing the amount of protein present, but it was decided to use non-ultracentrifuged extracts in future experiments, so that no important antigenic determinants were lost.

4.3.2 TREATMENT OF AQUEOUS PHENOL EXTRACTS WITH PROTEINASE K AND PERIODATE

Proteinase K destroyed unevenly-spaced bands in the S-LPS region, providing further evidence that these bands were protein and not S-LPS. Periodate destroyed the vast majority of the very high *Mr* (probably CP) and the very low *Mr* (lipid A and LPS

core) material, both of which would be expected to be very high in carbohydrate. Immunoblotting of Proteinase K- and periodate-treated extracts failed to show any material, suggesting that the treatments either reduced the antigenicity of the samples to such an extent that the Abs no longer recognised the epitopes, or that such a large amount of material was destroyed by the treatments that there was not enough left to be visualised by immunoblotting.

The common Ag was of particular interest in the treated extracts. To date, the structure of the *B. fragilis* common Ag has not been elucidated. In these experiments, it was destroyed by Proteinase K, which would suggest that it is made of protein. Further supporting evidence for this is that the common Ag was not present on silver-stained PAGE profiles of whole cell Proteinase K extracts of *B. fragilis* (Figure 10b). However, the defined common Ag band also disappeared from the periodate-treated extracts, and a larger, more diffuse band appeared immediately below. A similar pattern of results has also been obtained by immunoblotting of Proteinase K- and periodate-treated extracts using polyclonal and monoclonal Abs against the common Ag (S. Patrick, personal communication). Enterobacteria possess a common Ag, which is usually composed of a carbohydrate polymer of *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosaminouronic acid linked to membrane-bound fatty acids (Poxton 1993). Given the considerable differences between the other surface Ags of enterobacteria and *B. fragilis*, it is unlikely that their common Ags are similar in structure. If the *B. fragilis* common Ag was exclusively composed of carbohydrate, it would not have been destroyed by Proteinase K. At present, all that can be concluded from these results that the common Ag of *B. fragilis* is complex in structure, as are the rest of its surface Ags.

4.3.3 FRACTIONATION OF THE SURFACE POLYSACCHARIDE COMPLEX OF *BACTEROIDES FRAGILIS* NCTC 9343

The surface polysaccharide complex of *B. fragilis* NCTC 9343 was fractionated to try to separate out and purify the different components. Initially a partially purified aqueous phenol extract was separated into different components by gel filtration chromatography, which resulted in three different sets of material as visualised by PAGE and silver staining - high, intermediate and low *Mr*. On one of the gels (Figure 28b) a faint line was evident in the low *Mr* region in the same tracks as the high *Mr* material. Assuming that this was not an artefact, it suggests that the high *Mr* material was strongly linked to some low *Mr* material. The possibility that it was an artefact cannot be ruled out, as it was not present on the other gel (Figure 28a). Other bacterial capsules are known to be linked to lipid and anchored to the outer membrane (e.g. some *E. coli* capsules: Poxton & Arbuthnott 1990), and it is possible that this is also the case in *B. fragilis*.

Some of the high *Mr* material (assumed to be CP) was subjected to acid hydrolysis to split it into its two component polysaccharides - PSA and PSB. Chemical analysis was performed on the high, intermediate and low *Mr* material, and on PSA and PSB. Overall, the results from this were largely as would be expected: the high *Mr* material, PSA and PSB (all presumably CP) were high in carbohydrate, and the low *Mr* material (presumably lipid A and core oligosaccharide) was high in phosphorus and also fairly high in carbohydrate. The intermediate *Mr* fraction (possibly O-Ag) had some carbohydrate present, and also protein which could have been residual Pronase left over from the purification procedure, or protein from the original extract which had not been destroyed by Pronase, or both.

4.3.4 CHARACTERISATION OF THE SURFACE ANTIGENS OF *BACTEROIDES FRAGILIS* NCTC 9343 BY DOT BLOTTING AND IMMUNOBLOTTING

A crude aqueous phenol extract of *B. fragilis* NCTC 9343, and the surface polysaccharide fractions were examined using polyclonal and monoclonal Abs. Many results obtained from this study are not clear-cut and some are confusing, but this is probably a reflection of the complexity of the surface Ag complex of *B. fragilis*. The main points to be noted are listed below:

- PSB showed some reactivity with polyclonal and monoclonal Abs in dot blots, but it did not stain with silver and did not react with any Abs following PAGE. PSB is periodate-labile (Baumann *et al* 1992), so possible reasons why it was not visualised by silver staining or immunoblotting could have been that it was at too weak a concentration to stain - acid hydrolysis yields only very small amounts of PSB (A. Tzianabos, personal communication), or that it did not enter the separating gel.
- The intermediate and low *Mr* material, and PSA alone did not react with any Abs following PAGE, but did stain with silver. It is possible that the Ags on the gel and / or the Abs used to detect them were not sufficiently concentrated.
- PSA and PSB alone gave much poorer reactivity in dot blots and immunoblots than PSA and PSB together. It is possible that the acid hydrolysis and ion exchange chromatography, and / or the physical separation destroyed much of their antigenicity.
- Results from the dot blots and immunoblots allow some speculation as to what some of the epitopes of the uncharacterised MAbs may be: 2E12 could be specific for the LC or O-Ag, 3H5 and 1E2 could be specific for the O-Ag, and 2B8, 3C10 and 4C5 could be specific for the LC and EDL populations. However, this is only

speculative, and extensive further studies would need to be carried out before these could be confirmed.

- The high *Mr* material from the crude aqueous phenol extract, and the fractionated high *Mr* material both reacted with polyclonal rabbit serum against the LC of NCTC 9343 and with MAb 3D7 (specific for the LC and EDL populations). This confirmed that the high *Mr* material seen on PAGE profiles was, as suspected, CP and not LPS.

- MAb 6G3 produced one band only on the immunoblot, and the same single band was produced by polyclonal rabbit serum raised against the common Ag. This strongly suggests that 6G3 is specific for the common Ag, and further supporting evidence for this is that the same result has been obtained elsewhere using an independently prepared aqueous phenol extract of *B. fragilis* NCTC 9343 (S. Patrick, personal communication).

- MAb 3F6 (against an undefined non-capsular epitope in the SC population) did not react with any of the Ags in dot blots or immunoblots. The reason for this is that the cells for this experiment were grown in PPY, and as previously mentioned (Section 4.1.1) there do not appear to be any cells present in PPY which have antigenically small capsules. It was unfortunate that there was no MAb specific for the capsule of the SC population, as it would have been interesting to discover whether an anti-SC MAb reacted with PSA or PSB, to see whether these polysaccharides were present in the SC population. Conversely, it would have also been interesting to find out whether MAbs specific for PSA and PSB recognised LC, SC and EDL cells, and if so, to what extent. Studies such as these could perhaps shed more light on the diversity of the CP structures in *B. fragilis*.

The most striking observation from these studies, in agreement with previous work, is

the complex nature of the surface of *B. fragilis*. In some other bacteria the definition of LPS and capsule is unclear. For example in *E. coli* 0111 a high *Mr* surface polymer, located external to the LPS, consists of polymeric O-polysaccharide which is not linked to core or lipid A (Peterson & McGroarty 1985). It is also difficult to recognise a division between LPS and CP in *B. fragilis*. One of the major reasons for this is the within-strain variation of surface structures which makes consistent analysis extremely difficult, further complicated by the fact that many workers do not take this variation into consideration. Publications by Kasper and colleagues state that "pure CP" is obtained following fractionation and purification. However, one member of this group recently admitted that the CP which they prepare varies considerably from batch to batch in the relative amounts of PSA and PSB present. Moreover, it is invariably contaminated, to varying degrees from batch to batch, with LPS (A. Tzianabos, personal communication).

It has recently been suggested that "purified" CP from *B. fragilis* could be used as a vaccine to be administered prophylactically to patients at high risk of developing abscesses (Tzianabos *et al* 1995), as it has been previously shown that injection of *B. fragilis* CP into rats prevents subsequent abscess formation (Onderdonk *et al* 1982). In my opinion, this idea is somewhat over-ambitious, as it would be dangerous to administer any substance to a patient which is so variable in nature, and which is contaminated, to an unknown degree, with other substances including LPS.

In summary, despite much research and some slow progress, many unanswered questions about the surface of *B. fragilis*, and of course the other *Bacteroides* spp., remain. Further immunological studies may reveal more, but for a fuller understanding of this subject it will probably be necessary to carry out genetic studies, which at present are still in their infancy.

4.4 ANTIBODIES TO BACTEROIDES IN HEALTH AND DISEASE

To date, limited studies have been carried out on antibodies to bacteroides in human serum. Previous studies, carried out a long time before the genus *Bacteroides* was restricted to ten species, and before many of the species today classified as *Bacteroides* had been recognised, reported the presence of anti-bacteroides LPS Abs in serum from healthy individuals and from individuals with infections (reviewed by Hofstad [1979]). Experiments in this thesis were designed to build on these previous studies, and to try to discover whether bacteroides were significant in certain diseases (namely SIRS and IBD) where they had previously been assumed to be largely insignificant.

For many of the experiments in this study, in addition to measuring Abs to bacteroides LPS, Abs to a cocktail of R-LPS from three enterobacteria and *P. aeruginosa* were also measured. The aim of the study was not to make direct comparisons between bacteroides LPS and the R-LPS mixture, but rather to observe and compare the IgG responses in order to see whether that against bacteroides LPS was significant.

Non-ultracentrifuged aqueous phenol extracts of bacteroides LPS were used to measure IgG levels. It was known that these extracts were not pure LPS. However, it was decided not to try to purify the LPS further, as the aim was to mimic the *in vivo* situation as closely as possible; if hot phenol failed to separate the surface components of bacteroides completely, then the LPS is unlikely to be present in pure form in the body.

4.4.1 ANTI-BACTEROIDES LIPOPOLYSACCHARIDE IgG IN HEALTHY INDIVIDUALS

Every blood donor tested had anti-bacteroides and anti-enterobacterial / *Pseudomonas*

LPS IgG present in their serum. This suggests that healthy individuals are exposed to bacteroides LPS, and the most likely source of this is the gut. It is thought that anti-enterobacterial LPS Abs arise following exposure to gut-derived enterobacterial LPS (Barclay 1990, Windsor *et al* 1993, Barclay 1994), and it is therefore logical that anti-bacteroides LPS Abs would arise in the same way, especially when the differences in numbers of enterobacteria and bacteroides in the gut are taken into consideration. If LPS does translocate through the gut, then these results suggest that translocation must occur in healthy people, and they suggest that the gut is not the completely impenetrable barrier that it is often thought to be.

4.4.2 LEVELS OF ANTI-BACTEROIDES LIPOPOLYSACCHARIDE IgG IN HEALTHY INDIVIDUALS

The levels of anti-bacteroides LPS IgG in serum were highly variable between different individuals. In addition, when IgG was looked at in two different individuals by inhibition ELISA and immunoblotting (Sections 3.4.4 and 3.4.5), different IgG repertoires were seen, and individual # 2 appeared to have a greater amount of anti-bacteroides LPS IgG overall in their serum than individual # 1. This suggests that different individuals are exposed to bacteroides LPS to different extents. Assuming the source of the LPS is the gut, then the degree of exposure would be dependent on the degree of translocation. The degree of translocation would probably be dependent on lots of factors such as gut barrier function, diet, general state of health, age and previous illnesses.

Of note in one of the immunoblots of bacteroides LPS using human serum (Figure 36b) was a fine ladder pattern, characteristic of S-LPS, in *B. eggerthii*. This ladder pattern had not been observed by silver staining during the course of this thesis, although it has been observed by silver staining in a previous study (Maskell 1991).

4.4.3 CROSS-REACTIVITY OF ANTI-BACTEROIDES LIPOPOLYSACCHARIDE IgG

Inhibition ELISAs showed that whilst there were some epitopes shared between the different bacteroides LPSs, there were very few between bacteroides LPS and the enterobacterial / *Pseudomonas* LPS mixture. This is not surprising given that the lipid A structure of *B. fragilis*, and probably the other *Bacteroides* spp., is markedly different from that of the enterobacteria (Magnuson *et al* 1989, Lindberg *et al* 1990). Results from the inhibition experiments suggested that whilst there were some epitopes shared between the different bacteroides LPSs, each species had its own unique epitopes. This also appeared to be the case in the two *B. fragilis* strains tested, which is in keeping with previous studies showing limited cross-reactivity of MAbs raised against the CP of *B. fragilis* NCTC 9343 with the CP of other *B. fragilis* strains (Pantosti *et al* 1992, Pantosti *et al* 1993).

In the inhibition experiments, total absorption of all of the IgG was not achieved, not even when LPS derived from the same strain as the LPS coated onto ELISA strips was used for the absorptions. This could be to do with the accessibility of LPS in serum to Abs. LPS is notoriously difficult to detect in serum, and the reason for this is thought to be that it quickly becomes bound up in serum proteins, and as such becomes hidden from the immune system (Nelson 1991, Majde 1992). It is therefore possible that the LPS used to absorb out Abs became bound to serum proteins - for example high and low density lipoproteins (Wurtel *et al* 1994) and lipopolysaccharide binding protein (Tobias *et al* 1986, Tobias *et al* 1989) before all of the Abs had been absorbed out.

The graphs measuring correlation coefficients between IgG levels to different bacteroides LPSs (Figure 33) show a significant correlation in all cases except one. Whether the high level of correlation is due to the degree of exposure of individuals

to bacteroides LPS (i.e. if exposure to one Ag is high, exposure to another is correspondingly high and vice-versa), or due to a high degree of cross-reactivity of anti-bacteroides LPS IgG is a matter of debate. Both factors are probably involved. In some cases, a high correlation coefficient was observed together with a high level of inhibition (e.g. *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504; *B. caccae* MPRL 1555 and *B. thetaiotaomicron* NCTC 10582), suggesting a high degree of cross-reactivity, whereas in other cases a high correlation coefficient was obtained but the level of inhibition was very low (e.g. *B. variabilis* VPI 11368 and *B. eggerthii* NCTC 11155; *B. vulgatus* MPRL 1985 and *B. ovatus* MPRL 2370), suggesting that the high correlation was due to the degree of exposure of individuals to bacteroides LPS.

4.4.4 KINETICS OF ANTI-LIPOPOLYSACCHARIDE IgG RESPONSE IN SYSTEMIC INFLAMMATORY RESPONSE SYNDROME PATIENTS

In all SIRS patients, IgG levels against all Ags followed similar overall trends. In some cases, a sudden consumption of IgG was shown to be concurrent with an episode of endotoxaemia (confirmed previously by LAL assays [Scottish Sepsis Intervention Group, unpublished data]). In other cases, there was a decrease in IgG against all Ags but no endotoxin detected in the serum. However, as discussed above, endotoxaemia is often very transient and may not be detected in a LAL assay (Majde 1992), so IgG levels may prove a more accurate method of detecting it.

One of the most striking observations in this study was that in five out of six of the non-survivors, IgG levels against *B. fragilis* LPS were higher than levels of IgG against the other Ags. This only occurred in one survivor. Due to the small number of sepsis patients studied here, it is not possible to draw any firm conclusions linking certain Ab levels or kinetics to a fatal or non-fatal outcome. However, the study has demonstrated a definite IgG response to bacteroides LPS which was independent of,

but showed similar overall trends to, the response to enterobacterial LPS. Other recent studies have shown bacteroides LPS to be more biologically active in certain endotoxin assays than previously thought (Delahooke *et al* 1995a, Poxton & Edmond 1995). This, together with the nature of the IgG response, suggests that bacteroides LPS could be more important than previously thought in the development and outcome of SIRS.

It has recently been shown that in a TNF induction assay, when *B. fragilis* LPS is added in excess of *E. coli* LPS, rather than a cumulative effect, the *B. fragilis* LPS reduces the amount of TNF produced, with the amount produced being less than if *E. coli* LPS had been added alone (Delahooke *et al* 1995b). Bearing this in mind, an interesting hypothesis is that in fatal cases of SIRS, where anti-*B. fragilis* LPS IgG levels are high, the bacteroides LPS is being efficiently neutralised. Its neutralisation removes the immunomodulatory effect that it has on *E. coli* LPS, leaving the *E. coli* LPS to exert its full pathophysiological effects. In survivors, where levels of anti-*B. fragilis* LPS IgG are lower, the *B. fragilis* LPS is not as efficiently neutralised. Therefore, more *B. fragilis* LPS is present in serum, which exerts an immunomodulatory effect on *E. coli* LPS. Obviously this hypothesis is highly speculative, but if it was shown to be correct, it would have major implications in the management of SIRS patients. Moreover, measurement of anti-*B. fragilis* LPS IgG relative to other anti-LPS IgG could prove a useful means of identifying high risk patients.

4.4.5 ANTI-LIPOPOLYSACCHARIDE IgG IN INFLAMMATORY BOWEL DISEASE PATIENTS

Of the 28 IBD patients examined in this study, 26 had above median levels of IgG against all of the LPSs tested. This suggests that most people with IBD have more permeable gut walls than healthy individuals, which allow greater translocation of

bacteria and their products into the systemic circulation. A previous study found abnormally high levels of IgG1 in UC patients, and abnormally high levels of IgG2 in CD patients (MacDermott *et al* 1989). It would be interesting to measure IgG subclasses in the sera of the patients in this study, to see whether specific subclasses were being induced against given LPSs. Another study found high levels of total IgG in CD patients, but normal levels in UC patients (O'Mahony *et al* 1990). The present study found high levels of anti-LPS IgG in both CD and UC patients. However, no firm conclusions can be drawn from this as only two UC patients were included in the study.

In this study, serum IgG levels only were examined, as at the time these were the only samples available. However, previous studies have shown that it is better to look at the gut mucosal Ab levels in IBD rather than the serum Ab levels, as there is sometimes a lack of correlation between the systemic and the gut immune responses (O'Mahony *et al* 1990, O'Mahony *et al* 1991). Probably the easiest and most accurate way of examining gut Abs is to use whole gut lavage fluid, which is obtained by a relatively simple, non-invasive process using an orally-administered polyethylene glycol-based isotonic solution (O'Mahony *et al* 1990, Poxton *et al* 1995). It would have been useful if paired samples of serum and whole gut lavage fluid had been available from each patient, so that the systemic and mucosal immune responses could have been compared.

In addition to IgG, it would be interesting to look at levels of other Ab classes, both in serum and gut lavage fluid. Levels of IgA in the gut against bacteroides LPS and outer membranes have recently been shown to be significantly raised in people with CD (Poxton *et al* 1995). It would be very interesting to look at IgE levels, especially in the gut, against bacteroides and other Ags, as excessive IgE is produced in hypersensitivity reactions. Therefore, if an allergic reaction was occurring in the gut

against, for example, bacteroides LPS, then IgE levels against bacteroides LPS would be expected to be elevated.

In summary, this study showed raised serum anti-LPS IgG levels in 26 of 28 IBD patients, which suggests that LPS could be translocating from the gut to a greater degree in people with IBD than in healthy individuals. Due to the small number of serum samples used in this study and the lack of gut lavage fluid samples, it is not possible to draw any other conclusions. However, the results obtained do create the possibility that bacteroides could be involved in the pathogenesis of IBD, and indicate that the study would be worth extending.

CONCLUSIONS

- Growth environment often has a profound effect on cell surface Ag expression in *Bacteroides* spp.
- Growth environment has a major influence on the susceptibility of certain *Bacteroides* strains to the bactericidal effects of serum complement.
- In one *B. fragilis* strain, complement resistance could be due, at least in part, to the expression of S-LPS.
- All healthy adults appear to have anti-bacteroides LPS IgG in their serum, with levels varying from individual to individual. The anti-bacteroides LPS IgG is not simply anti-enterobacterial LPS IgG which cross-reacts with bacteroides LPS; the two are independent of each other.
- Certain trends in anti-bacteroides LPS IgG kinetics in SIRS patients have been demonstrated, which suggest that bacteroides LPS could be significant in SIRS.
- Anti-bacteroides LPS IgG has been found, often in high levels, in the serum of people with IBD, suggesting that bacteroides could be significant in the pathogenesis of this disease.
- Work carried out on the surface polysaccharide complex of *B. fragilis* has revealed a very complex situation, in agreement with previous studies. Much more work needs to be carried out in this area before any firm conclusions can be drawn.

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The influence of growth medium on serum sensitivity of *Bacteroides* species

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Summary. The susceptibility of 12 different *Bacteroides* strains (representing nine species) to the bactericidal effect of human serum complement was investigated. When grown in nutrient-rich proteose peptone-yeast extract medium, all 12 strains were, to varying degrees, sensitive to serum. However, when grown in Van Tassel and Wilkins's minimal medium, six of the 12 strains became markedly more serum resistant. Five of these six strains became totally resistant to serum when grown in heat-inactivated (56°C, 30 min) sheep serum. By Percoll discontinuous density centrifugation and light microscopy, the ratio of bacteria with large and small capsules was found to vary with the growth medium used. Lipopolysaccharide (LPS) was extracted with aqueous phenol after growth in the three media. Polyacrylamide gel electrophoresis (PAGE) and silver staining of the LPS showed some differences in LPS profiles in all strains tested. Therefore, variation of growth conditions results in alterations of both the expression of surface structures and, in some cases, sensitivity to serum. The biochemical basis for these changes requires further investigation.

Introduction

Members of the genus *Bacteroides* (formerly the *Bacteroides fragilis* group) are common components of the healthy colonic flora and are often involved in both pure and mixed infections in man—e.g., intra-abdominal abscesses, bacteraemia, wound and urogenital infections. *B. fragilis*, the type species, is the anaerobe isolated most commonly from clinical specimens, and the most common cause of anaerobic bacteraemia.^{1–4} The surface polysaccharides of the *B. fragilis* group are widely considered to be major virulence determinants.^{5,6} However, despite extensive research, there is still debate as to whether the lipopolysaccharide (LPS) of *B. fragilis* is rough⁵ or smooth⁷ and the roles of the capsule and LPS as virulence factors are unclear. This is further confused by the recognition, which has not been considered by many workers, that following fractionation by Percoll discontinuous density centrifugation,⁸ a wild-type laboratory culture of *B. fragilis* has been shown to be morphologically heterogeneous with respect to both size⁹ and antigenicity^{10,11} of its capsule.

Resistance to the bactericidal effects of serum complement has been clearly shown to be associated with virulence in a wide range of species.^{12,13} In some cases, the mechanism of resistance is known, and both the O polysaccharide of LPS and capsular polysaccharide (CP) have been implicated as important virulence determinants. For example, wild-type *Sal-*

monella minnesota (with smooth LPS) is highly resistant to complement, whereas the rough mutant is extremely sensitive.¹⁴ In the case of *Escherichia coli* K1 strains, the CP is responsible for complement resistance.¹⁵

Previous studies have shown that *Bacteroides* strains isolated from infections are generally more resistant to complement than those isolated from faeces.¹⁶ Clinical isolates of *B. fragilis* have been found to be more resistant to complement than clinical isolates of other *Bacteroides* species.¹⁷ Also, complement-resistant strains of *B. fragilis* have been shown to survive better than complement-sensitive strains in a subcutaneous model of infection.¹⁸ However, the mechanism of complement resistance in *Bacteroides* strains is un-

Table 1. *Bacteroides* strains

Species	Strain no.	Source
<i>B. fragilis</i>	MPRL 1504	Wound
<i>B. fragilis</i>	NCTC 9343	Appendix abscess
<i>B. uniformis</i>	ATCC 8492	Unknown
<i>B. vulgatus</i>	MPRL 1985	Faeces
<i>B. vulgatus</i>	MPRL 1651	Faeces
<i>B. caccae</i>	MPRL 1555	Wound
<i>B. ovatus</i>	MPRL 2370	Blood
<i>B. eggerthii</i>	NCTC 11155	Faeces
<i>B. distasonis</i>	ATCC 8503	Unknown
<i>B. thetaiotaomicron</i>	MPRL 1959	Blood
<i>B. thetaiotaomicron</i>	NCTC 10582	Faeces
<i>B. variabilis</i>	VPI 11368	Unknown

MPRL, departmental stock culture; NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; VPI, Virginia Polytechnic Institute, USA.

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known, but CP is not thought to enhance resistance,¹⁹ while the role of LPS is unclear.

It is well recognised that the growth environment of bacteria greatly influences the phenotypic expression of surface molecules. This can be fundamental in the adaptive process that enables an invading pathogenic bacterium to survive.^{20,21} The aims of this study were: to investigate whether different growth conditions affected the sensitivity of *Bacteroides* spp. to serum; to determine whether any change in sensitivity was concomitant with a change in surface chemistry; and to investigate whether different methods of processing serum affected its bactericidal capabilities.

Materials and methods

Bacteria and growth conditions

The strains used and their source are listed in table I. Bacteria were grown to early stationary phase in nutrient-rich proteose peptone-yeast extract medium (PPY),²² Van Tassell and Wilkins's minimal medium (VT and W)²³ and heat-inactivated (56°C, 30 min) sheep serum (HISS). Cultures were incubated at 37°C in an atmosphere of H₂ 10%, CO₂ 10% and N₂ 80% in an anaerobic cabinet (Forma), and were checked for purity by Gram's stain and by aerobic and anaerobic incubation on Columbia Blood Agar (Oxoid).

Collection of serum

Human serum, used as a source of complement, was collected from five healthy adult volunteers in two ways as follows. (A) Freshly drawn blood was allowed to clot at 37°C for 30 min, then centrifuged at 4000 *g* for 10 min. Sera were removed, centrifuged as before and the supernates were pooled and stored at -70°C in 1-ml volumes until just before use. (B) Freshly drawn blood was left to clot overnight at room temperature. It was then placed at 4°C for 30 min before removal of serum. The sera were centrifuged at 4000 *g* for 10 min before the supernates were pooled and stored at -70°C in 1-ml volumes until just before use.

The haemolytic complement value (CH50) of the individual and pooled serum samples was checked as described previously²⁴ except that phosphate-buffered saline (PBS; Oxoid) was used instead of barbitone-buffered saline. Frozen specimens were thawed only once.

Serum sensitivity assay

Bacteria washed once in complement fixation test buffer (CFTB; Oxoid) were resuspended to a concentration of *c.* 10⁵ cfu/ml in either CFTB only (control), CFTB + serum 10% or CFTB + serum 40% and incubated aerobically (2 ml in 2.5-ml closed plastic tubes) at 37°C for 2 h with end-over-end rotation. Samples (100 µl) were taken at 0, 1 and 2 h, diluted 1 in 50 in CFTB, and 100 µl of the resulting suspension were spread on Columbia blood agar in duplicate.

After anaerobic incubation for 48 h, colonies were counted and the percentage survival compared to the control was calculated. All experiments were repeated at least twice. As a further control, all bacteria found to be sensitive to serum were resuspended to 10⁵ cfu/ml in CFTB + heat-inactivated (56°C, 30 min) human serum 40% and treated as above. In a preliminary experiment complement-mediated killing in an anaerobic environment with reduced (anaerobic) buffers was compared with the aerobic system described above. No differences were seen in bacterial survival.

Percoll gradients

Cell capsulation was assessed by Percoll (Pharmacia) discontinuous density centrifugation. Percoll was diluted as described previously,⁸ and a step gradient was produced by layering 1-ml volumes of 80% (bottom), then 60%, 40% and 20% (top) Percoll into 70 × 20-mm glass test tubes. A sample of an early stationary phase culture of the test organism (1.25 ml) was applied to the top of the 20% layer and the gradient was centrifuged at 2600 *g* for 20 min.

LPS preparation

LPS was prepared from washed cells obtained from a 10-ml early stationary phase culture by the micro-method developed by Fomsgaard *et al.*,²⁵ which was based on the aqueous phenol method of Westphal and Luderitz.²⁶

PAGE

PAGE was performed on acrylamide 14% w/v slab gels with the Laemmli buffer system,²⁷ except that SDS was omitted from the stacking and separating buffers. Samples (20 µl) of the aqueous phenol LPS extracts were loaded on the gels and the separating gel was stained with silver by the method developed by Tsai and Frasch,²⁸ as modified by Hancock and Poxton.²⁹

Results

Comparison of two methods of serum processing

Human serum processed by methods A and B was tested for its ability to kill *B. fragilis* MPRL 1504, *B. fragilis* NCTC 9343 and *B. vulgatus* MPRL 1985 grown in PPY (table II). With all three strains, serum processed by method A was more bactericidal than that processed by method B. The CH50 values of the sera (method A = 63.1 units/ml, method B = 49.1 units/ml) showed complement activity to be better preserved in serum processed by method A. Therefore, in all subsequent experiments, serum processed by method A was used.

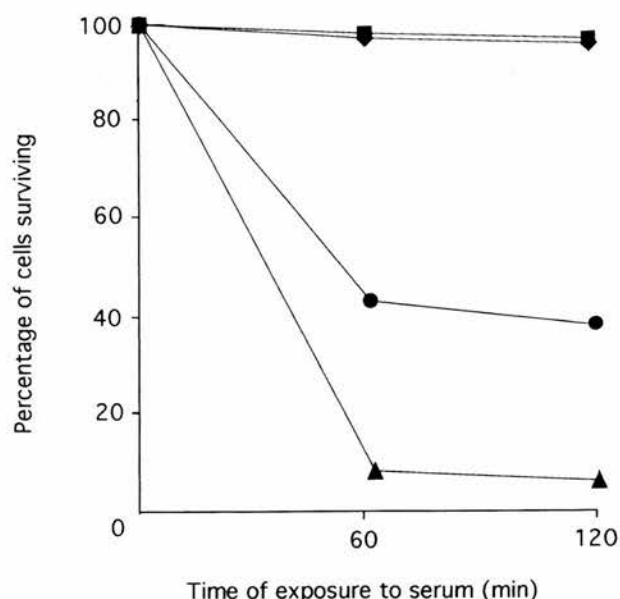
Serum sensitivity of 12 Bacteroides strains grown in three different media

The 12 *Bacteroides* strains listed in table I were grown in three different media (PPY, VT and W, and

Table II. Complement killing of three *Bacteroides* strains grown in PPY by serum processed by two different methods

Strain	Assay medium	Percentage of cells surviving* with	
		Method A (CH50 = 63.1)	Method B (CH50 = 49.1)
<i>B. fragilis</i> MPRL 1504	CFTB only (control)	95.2	94.6
	10% serum	8.9	16.1
	40% serum	7.8	10.1
<i>B. fragilis</i> NCTC 9343	CFTB only (control)	95.3	93.8
	10% serum	55.1	77.6
	40% serum	14.2	37.3
<i>B. vulgatus</i> MPRL 1985	CFTB only (control)	91.9	93.0
	10% serum	56.2	68.3
	40% serum	49.8	58.3

*Survival after 1 h compared to time 0.

**Fig. 1.** Survival of *B. fragilis* MPRL 1504 in 40% human serum after growth in three media: ■—■ control (no serum); ▲—▲ PPY; ●—● VT and W; ◆—◆ HISS. Points shown are mean percentage survival calculated from four replicates.

HISS) and then tested for their ability to survive in 10% human serum, 40% human serum and buffer only (control). Table III shows the results for all 12 strains with 40% serum. When grown in PPY, all strains were sensitive to serum to varying degrees. However, when grown in VT and W, six of the 12 strains (*B. fragilis* MPRL 1504, *B. fragilis* NCTC 9343, *B. caccae* MPRL 1555, *B. thetaiotaomicron* MPRL 1959, *B. ovatus* MPRL 2370 and *B. vulgatus* MPRL 1985) became markedly more resistant to serum. With the exception of *B. vulgatus* MPRL 1985, these strains became totally resistant to serum when grown in HISS. In every case, survival in CFTB alone was between 90 and 100%. Fig. 1 shows the survival of *B. fragilis* MPRL 1504 in serum after growth in the three

Table III. Survival of 12 *Bacteroides* strains after 1 h in 40% human serum following growth in three media

Strain no.	Percentage survival of bacteria grown in		
	PPY	VT and W	HISS
<i>B. fragilis</i> MPRL 1504	7.8	37.9	92.2
<i>B. fragilis</i> NCTC 9343	14.2	68.1	94.2
<i>B. caccae</i> MPRL 1555	10.2	48.9	91.3
<i>B. ovatus</i> MPRL 2370	74.4	85.6	98.1
<i>B. thetaiotaomicron</i> MPRL 1959	52.9	71.8	97.6
<i>B. thetaiotaomicron</i> NCTC 10582	23.3	19.7	25.1
<i>B. uniformis</i> ATCC 8492	13.0	11.3	10.6
<i>B. vulgatus</i> MPRL 1985	49.8	61.1	41.5
<i>B. vulgatus</i> MPRL 1651	14.4	21.5	15.5
<i>B. eggerthii</i> NCTC 11155	0.0	3.2	0.0
<i>B. distasonis</i> ATCC 8503	6.7	0.0	2.6
<i>B. variabilis</i> VPI 11368	0.0	0.0	1.2

media. Heat inactivation of serum destroyed bactericidal activity in every case where a strain was killed by active serum.

Capsulation of cells in three media

A step gradient of Percoll was used to assess the degree of capsulation of the 12 *Bacteroides* strains grown in three different media. All of the strains (except *B. eggerthii* NCTC 11155 which was non-capsulate) had a characteristic ratio of cells with large:small:no capsule, and in all cases, this ratio varied with the growth medium. The Percoll gradients of *B. fragilis* MPRL 1504 after growth in the different media are shown in fig. 2a. India ink smears of the three cell types of *B. fragilis* MPRL 1504 (i.e., large, small, no capsule), extracted from the Percoll gradients, are shown in fig. 2b.

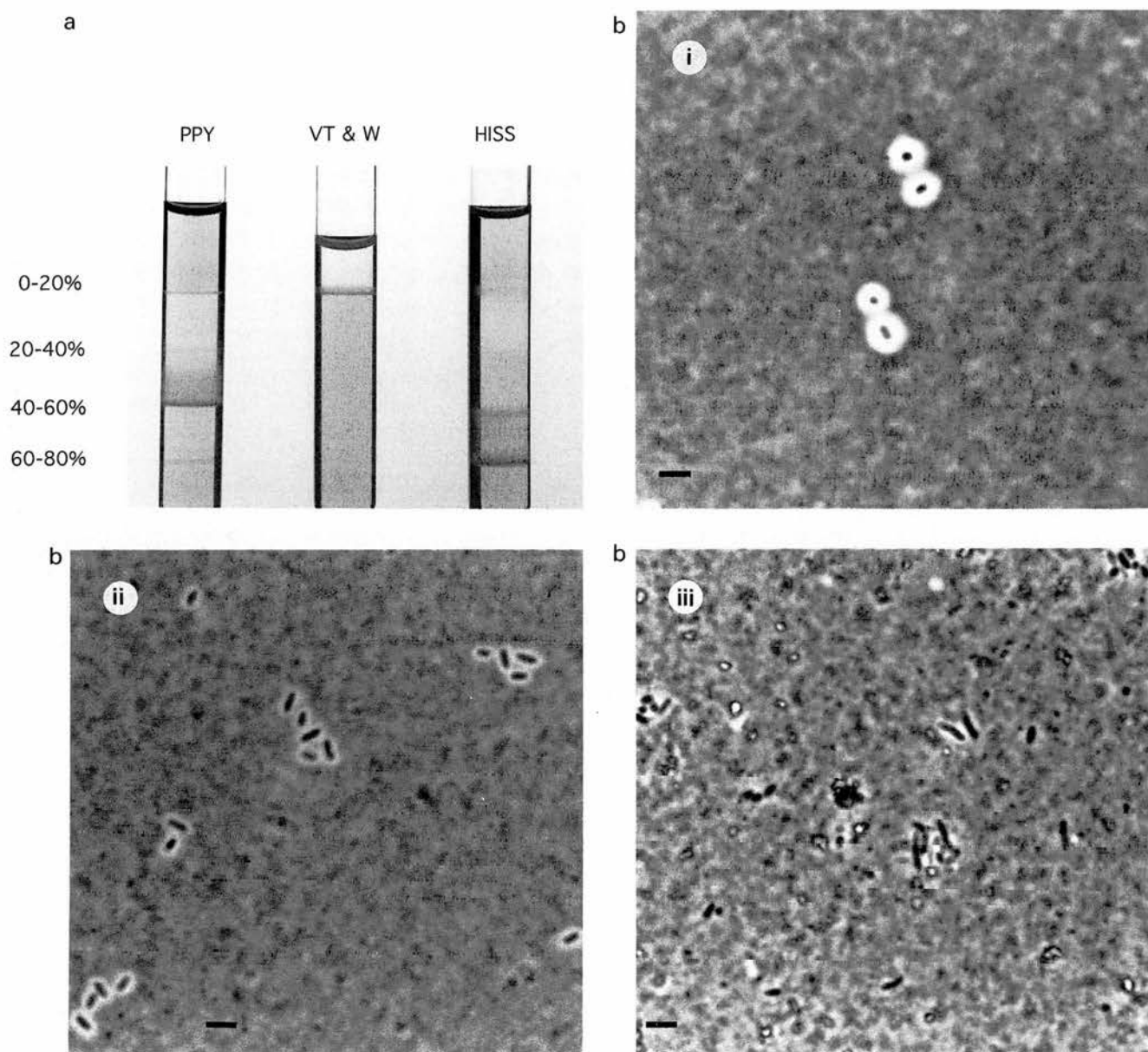


Fig. 2. a, four-step (20, 40, 60 and 80 %) Percoll density gradients after centrifugation with cultures of *B. fragilis* MPRL 1504 grown in three media. Cells with large capsules were found at the 0–20 % interface, those with small capsules at the 20–40 % and 40–60 % interfaces and those with no capsule at the 60–80 % interface. b, photomicrographs of *B. fragilis* MPRL 1504 showing bacteria with: i, large capsules (from VT and W); ii, small capsules (from PPY); iii, no capsules (from HISS). Cells were removed from the most concentrated band in each of the Percoll gradients and stained with India ink. Bar markers = c. 2 μm.

LPS profiles of bacteria grown in three media

The LPS profiles of all 12 *Bacteroides* strains grown in the three media were examined by aqueous phenol extraction followed by PAGE and silver staining. Fig. 3 shows the silver-stained PAGE profiles of the LPS of the six strains that showed most altered sensitivity to serum in different media. Each species had a characteristic profile, with some obvious differences seen in that of any given strain when grown in different media. *B. vulgatus* was the only species to show a distinct ladder pattern that is characteristic of smooth LPS as seen in enterobacteria, and this strain showed the least differences in staining pattern. Other profiles were similar to those published previously,^{7,30} with low M_r bands characteristic of rough or semi-rough

strains, but all also had a few strongly staining high M_r bands which we have shown previously to be made up of closely spaced ladder patterns.⁷ The apparent weak staining of *B. fragilis* MPRL 1504 (tracks 4–6) is probably because this is an extremely mucoid strain and much of the extract may be of such a high M_r that it is unable to enter the polyacrylamide gel. There is no obvious correlation between pattern and change in sensitivity to serum.

Discussion

The mode of action of the complement system is now largely understood, and some bacterial resistance mechanisms to its lethal effects are known. However, a major drawback in experimental work on this system

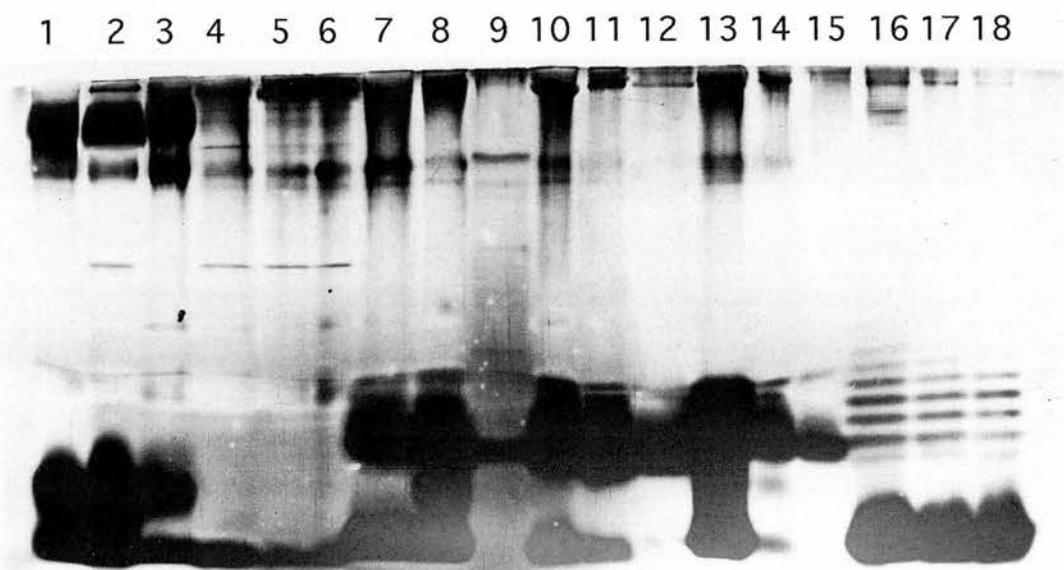


Fig. 3. Silver-stained PAGE (14% acrylamide) LPS profiles of aqueous phenol extracts of six *Bacteroides* strains following growth to early stationary phase in PPY, HISS and VT and W. Tracks: 1, *B. fragilis* NCTC 9343 (PPY); 2, *B. fragilis* NCTC 9343 (VT and W); 3, *B. fragilis* NCTC 9343 (HISS); 4, *B. fragilis* MPRL 1504 (PPY); 5, *B. fragilis* MPRL 1504 (VT and W); 6, *B. fragilis* MPRL 1504 (HISS); 7, *B. caccae* MPRL 1555 (PPY); 8, *B. caccae* MPRL 1555 (VT and W); 9, *B. caccae* MPRL 1555 (HISS); 10, *B. ovatus* MPRL 2370 (PPY); 11, *B. ovatus* MPRL 2370 (VT and W); 12, *B. ovatus* MPRL 2370 (HISS); 13, *B. thetaiotaomicron* MPRL 1959 (PPY); 14, *B. thetaiotaomicron* MPRL 1959 (VT and W); 15, *B. thetaiotaomicron* MPRL 1959 (HISS); 16, *B. vulgatus* MPRL 1985 (PPY); 17, *B. vulgatus* MPRL 1985 (VT and W); 18, *B. vulgatus* MPRL 1985 (HISS).

is that there is no standardised method for testing serum sensitivity, and also no universal definition of serum sensitivity.¹³ Many papers do not specify how serum used in sensitivity experiments was processed and stored. This study has shown that different methods of processing serum affect complement activity (and therefore bacterial killing), and so may significantly affect the outcome of an experiment and the conclusions drawn from it.

Previous studies investigating the susceptibility of *Bacteroides* spp. to serum have done so after growth in one medium only. However, this study has shown dramatic changes in the sensitivity of some *Bacteroides* strains to serum when growth medium is altered. These results demonstrate a need to take growth medium into consideration when assessing serum sensitivity of bacteria, and also possibly when assessing other virulence factors.

All the *Bacteroides* strains in this study that became totally resistant to serum when grown in HISS (table II) were of clinical origin (table I). This is in agreement with a previous study by Casciato *et al.*,¹⁶ who found that faecal isolates of *Bacteroides* were significantly more sensitive to serum than those isolated from clinical infections. Resistance to complement-mediated killing is a well recognised virulence factor and is usually associated with the presence of a capsule or smooth LPS. This relationship does not seem to hold for the *Bacteroides* strains investigated here. *B. fragilis* MPRL 1504 grown in HISS was non-capsulate, and was resistant to complement, whereas the cap-

sulate cells (grown in PPY and VT and W) were sensitive. This is in agreement with a previous study, where Reid and Patrick¹⁹ found the non-capsulate variant of *B. fragilis* ATCC 23745 to be resistant to complement, and the capsulate variant to be sensitive. Of the other four strains in this study that became resistant to serum after growth in HISS, two were mainly non-capsulate and two were capsulate. Although differences were seen in the LPS patterns of any given strain when grown in three different media, we were unable to relate changes in pattern to changes in sensitivity to serum. However, the resolution of *Bacteroides* LPS with silver staining tends to be much poorer than that of many other organisms (e.g., the Enterobacteriaceae).^{21,31} Also, we suspect that certain high M_r polysaccharides may not be entering the gel and it is, therefore, possible that there were differences that were undetected by this method. *B. vulgatus* (with smooth LPS) remained sensitive to serum, suggesting that smooth LPS alone is not responsible for serum resistance in *Bacteroides*.

Although it has been demonstrated that variation in culture conditions can markedly affect sensitivity to serum complement, the biochemical basis is still uncertain. This can be resolved only when the surface chemistry of *Bacteroides* strains is understood more fully.

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Anti-bacteroides lipopolysaccharide IgG levels in healthy adults and sepsis patients

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Abstract

Members of the genus *Bacteroides* greatly outnumber enterobacteria in the human colon and therefore represent a vast potential pool of biologically active LPS. An enzyme-linked immunosorbent assay was developed to estimate the distribution of IgG levels to LPS from *B. fragilis*, *B. vulgatus*, *B. thetaiotaomicron* and to a mixture of rough LPS from three enterobacteria and *Pseudomonas aeruginosa* in sera from 641 adult blood donors. By inhibition ELISA some cross-reactivity was demonstrated between the different anti-bacteroides LPS IgG, but with very little between the anti-bacteroides LPS IgG and the anti-enterobacterial/*Pseudomonas* LPS IgG. Serum IgG was measured daily over 5–9-day periods in 12 sepsis patients (6 survivors, 6 non-survivors) and in a healthy individual. In all patients IgG levels fluctuated to a greater extent than levels in a healthy subject. Variations all followed similar overall trends and indicated that exposure to bacteroides LPS had occurred. In 5 out of 6 survivors, IgG levels were rising at the end of the period, while 4 of the 6 non-survivors showed falls, with an exception showing increasing levels to *B. fragilis* LPS. In 5 out of 6 non-survivors, IgG levels against *B. fragilis* LPS were substantially higher than those against the other LPSs. In this small sample some trends in antibody kinetics have been recognised which suggest bacteroides LPS may be significant in sepsis, and indicate that this study should be extended.

Keywords: Bacteroides; Lipopolysaccharide; Sepsis; Enzyme-linked immunosorbent assay; IgG

1. Introduction

Despite considerable recent advances in technology and drug therapy, systemic inflammatory response syndrome or 'sepsis' remains the most common cause of death in critically ill intensive care patients [1,2]. Endotoxin (lipopolysaccharide; LPS)

plays a central role in the development of sepsis by inducing cascades of cytokines, complement and clotting factors. In severe cases, this will result in shock, multiple organ failure and a mortality of up to 90% [3].

LPS may enter the circulation either directly from a site of infection or by translocation across an ischaemic gut wall [4,5]. Previous studies have shown Gram-negative bacteraemia to be present in only 21–37% of sepsis cases [6], and it is thought that in patients where there is no obvious Gram-negative

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infection, the source of LPS is the gut [7]. It has previously been assumed that, in those cases where LPS is gut-derived, it is that of the enterobacteria (particularly *Escherichia coli*) which is responsible for the pathophysiological effects of sepsis, with other gut organisms not thought to be significant [8].

The outcome of an invasion by any foreign material into the circulation is largely dependent on the timing and the magnitude of the antibody response mounted against it. Barclay and colleagues have carried out extensive studies investigating the magnitude and kinetics of serum antibody response to LPS core from enterobacteria and closely related species in sepsis patients. It was found that non-survivors were more likely to have low endogenous levels of IgG which failed to recover. In both survivors and non-survivors, a sudden consumption of antibody appeared to be indicative of a recent episode of endotoxaemia (often confirmed by limulus amoebocyte lysate gelation (LAL) assays), and as such was a useful indirect method of showing when endotoxaemia had occurred [9–12].

Members of the genus *Bacteroides* (formerly the *Bacteroides fragilis* group) constitute 20–30% of the colonic microbial flora [13] and are often involved in both pure and mixed infections in man. *B. fragilis*, the type species, is the most frequently isolated anaerobe from clinical specimens, and the most common cause of anaerobic bacteraemia [14,15]. Anaerobes outnumber enterobacteria in the colon by approximately 1000 to 1 [13].

Bacteroides LPS has previously been found to be 100- to 1000-fold less biologically active than enterobacterial LPS in various in vivo and in vitro assays [16]. However, given the differences in numbers between *E. coli* and *Bacteroides* in the gut, there is potentially as much if not more biological activity from bacteroides LPS as there is from that of *E. coli*.

To date, no data have been published with regard to serum antibody levels to *Bacteroides* spp., either in healthy individuals or in sepsis patients. The aims of this study were therefore: (i) to develop an enzyme-linked immunosorbent assay (ELISA) measuring anti-bacteroides LPS IgG in serum which could then be used indirectly to assess LPS levels in serum; (ii) to use the assay to determine the distribution of anti-bacteroides LPS IgG within a normal blood donor population; (iii) to use the assay to

Table 1

Bacterial strains

Species	Strain number
<i>Bacteroides fragilis</i>	NCTC 9343
<i>Bacteroides vulgatus</i>	MPRL 1985
<i>Bacteroides thetaiotaomicron</i>	NCTC 10582
<i>Escherichia coli</i> K12	MPRL 1303
<i>Salmonella typhimurium</i> Rc 878	MPRL 0947
<i>Klebsiella pneumoniae</i> 10B	MPRL 0954
<i>Pseudomonas aeruginosa</i> PAC 611	MPRL 1091

NCTC = National Collection of Type Cultures, UK; MPRL = Departmental stock culture.

follow the kinetics of anti-bacteroides LPS IgG in sepsis patients where the source of LPS is likely to be the gut (and where consumption of antibody would suggest exposure to LPS); (iv) to compare the kinetics of anti-bacteroides LPS IgG with the kinetics of anti-enterobacterial LPS IgG; and (v) to try to determine from these results whether bacteroides LPS plays a significant part in the course and the outcome of sepsis.

2. Materials and methods

2.1. Bacteria and growth conditions

Bacterial strains used are listed in Table 1. *Bacteroides* strains were grown in 1-l batch cultures to early stationary phase at 37°C in an atmosphere of H₂ 10%, CO₂ 10% and N₂ 80% in an anaerobic cabinet (Forma), in proteose peptone-yeast extract medium [17]. All other bacteria were grown aerobically at 37°C in 2-l batch cultures in nutrient broth (Gibco). All cultures were checked for purity by Gram's staining and aerobic and anaerobic incubation on Columbia blood agar (Oxoid). Cells were harvested by centrifugation (10 000 × g, 15 min), washed twice in phosphate-buffered saline (PBS, Oxoid) and lyophilised.

2.2. Serum used in study

A random selection of healthy adult (blood donor) sera was obtained from the South East Scotland Regional Blood Transfusion Centre, Edinburgh.

Serum samples were obtained from sepsis patients in a study conducted by the Scottish Sepsis Interven-

tion Group (paper submitted). Approval was obtained from the relevant Ethical Committees and informed consent obtained from all patients.

All sera were stored at -20°C until use.

2.3. LPS preparation and coating onto ELISA strips

Bacteroides strains

Lyophilised LPS (prepared by the aqueous phenol method of Westphal and Luderitz [18] as described by Hancock and Poxton [19]) was complexed with polymyxin B after the method of Scott and Barclay [20]. LPS was resuspended in pyrogen-free (PF) H_2O to 1 mg/ml, and an equal volume of a 1 mg/ml solution of polymyxin B sulphate (Sigma) was added. The mixture was sonicated for 30 s at 10 μ (MSE Soniprep), stirred for 90 min at 20°C , then re-sonicated as above. Following dialysis for 18 h (Spectra pore membrane MWCO 2000) against PF H_2O at 4°C , the mixture was diluted 1:50 in ELISA coating buffer: 0.05 M carbonate/bicarbonate, pH 9.6, containing 0.02% sodium azide. ELISA strips (Immunomodule polysorp F8 Nunc, Intermed) were coated at 100 μl /well and incubated at 20°C overnight. The strips were washed four times with ELISA wash buffer: PBS, pH 7.4 containing Tween 20 (0.05% w/v, Sigma) and sodium azide (0.05% w/v). They were then post-coated with bovine serum albumin (BSA, 5%w/v, ICN) and sodium azide (0.02% w/v) in PBS at 100 μl /well and were incubated as above. Finally the strips were washed four times, rinsed once in PF H_2O and stored at -20°C until use.

Other bacterial strains

A mixture of equal weights of *E. coli*, *S. typhimurium*, *K. pneumoniae* and *P. aeruginosa* rough LPS extracted using the phenol/chloroform/petroleum ether method of Galanos et al. [21] was resuspended in PF H_2O to 1 mg/ml and complexed with polymyxin B sulphate and coated onto ELISA strips as above.

2.4. ELISA

A 1 in 200 dilution of the serum samples to be tested was made in ELISA dilution buffer: PBS

containing Tween 20 (0.05% w/v), BSA (0.5% w/v) and polyethylene glycol M_r 6000 (4% w/v, Sigma). The diluted serum (100 μl) was added to each well on the appropriate LPS-coated ELISA strips and incubated for 90 min at 37°C before washing four times. Alkaline phosphatase-conjugated anti-human IgG (Sigma) was diluted in ELISA dilution buffer 1 in 1000 and added at 100 μl /well. The strips were incubated and washed as above. Alkaline phosphatase substrate (1 mg/ml p-nitrophenyl phosphate (Sigma) in 0.05 M carbonate/bicarbonate buffer pH 9.8 containing 1 mM MgCl_2) was added at 100 μl /well. The strips were incubated at 20°C for 30–40 min, and the absorbances were read at 405 nm in an ELISA plate reader (Anthos 2001, Labtec). A negative control was included where the serum was replaced by pure diluent, and the absorbance of the negative control was subtracted from each of the test wells.

2.5. Inhibition ELISA

This was carried out as above, except that after the serum was diluted 1 in 200, LPS was added to a final concentration of 1 mg/ml, and this was incubated at 37°C for 30 min prior to carrying out the ELISA. Control serum was treated identically except no LPS was added. Serum used in this case was pooled from 10 random blood donors.

2.6. Determination of the distribution of anti-LPS IgG levels within blood donor population

Initially a sample of 50 random blood donors was screened by ELISA for IgG levels to *B. fragilis* NCTC 9343, *B. vulgatus* MPRL 1985, *B. thetaio-taomicron* NCTC 10582 and the rough LPS mixture. Serum containing close to median levels of IgG to all of the antigens was selected as a standard. A further 641 donors were then screened, and to standardise the optical density (OD) readings, on each plate a sample of the standard serum was also tested. The OD of this serum was given an arbitrary value of 100% in each case, and the ODs of the 641 sera were converted to percentages using the standard serum. The median levels of IgG to all of the antigens within the 641 donors were calculated.

2.7. Determination of anti-LPS IgG in sepsis patients

ELISAs were performed on sequential serum samples from 12 sepsis patients. To standardise OD readings, another serum was selected as a standard from the 641 screened expressing close to median levels of IgG to all of the antigens and used as above. As a control, serum taken from a healthy individual on 6 consecutive days was also tested.

3. Results

3.1. Determination of average levels of anti-LPS IgG in 641 random blood donors

Sera from 641 blood donors were screened for IgG against aqueous phenol extracts of *B. fragilis*,

B. vulgatus and *B. thetaiotaomicron*, and against a mixture of rough LPS from three enterobacteria and *P. aeruginosa*. The results of this screen are shown on Fig. 1. Distributions of anti-LPS IgG in the donor population tested were normal in appearance on histograms, except anti-*B. fragilis* IgG which showed a slight negative skew.

3.2. Estimation of cross-reactivity of antibodies

An inhibition ELISA was used to determine the degree of cross-reactivity of the antibodies to the different LPSs. This involved pre-incubating pooled blood donor serum with the different LPSs to try to neutralise any cross-reactivity prior to using it in ELISA, and measuring the reduction in OD compared to a control which was not pre-incubated with

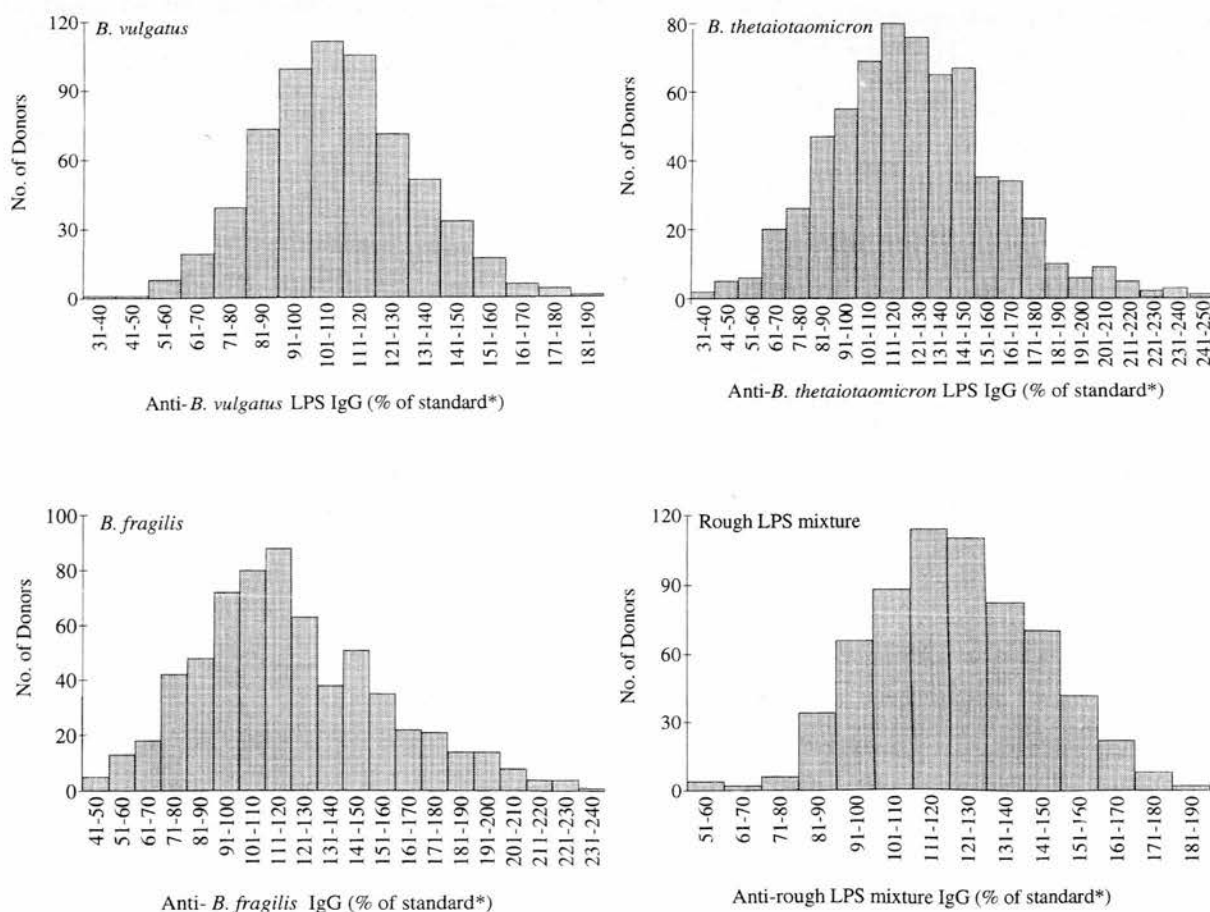


Fig. 1. Distribution of anti-LPS IgG in 641 blood donors by quantitative screening ELISA. Serum from a donor expressing close to median levels of IgG to all LPSs tested, selected from a preliminary screen of 50 random blood donors was included on all plates to standardise OD readings. Each donor serum was tested in duplicate. * Standard = 100%

LPS. The results of this are shown on Table 2. Pre-incubation of serum with homologous LPS reduced the OD readings to between 2.4 and 12.7% of the control (i.e. approx. 87–97% of the IgG was absorbed out against any given LPS). The greatest amount of cross-reactivity was between IgG reacting with *B. vulgatus* and *B. thetaiotaomicron*, and there was limited cross-reactivity between IgG reacting with *B. vulgatus* or *B. thetaiotaomicron* and *B. fragilis*. Cross-reactivity between IgG against any of the bacteroides LPSs and the enterobacterial/*Pseudomonas* rough LPS mixture was very limited.

3.3. Kinetics of anti-LPS IgG response in sepsis patients

ELISAs were performed on sera taken daily over 5- to 9-day periods from 12 sepsis patients (6 survivors, 6 non-survivors) with a clinical diagnosis of abdominal sepsis and blood culture negative in whom LPS was thought to be gut derived. Although no cases were identical, several broad observations could

be made on the overall trend of results, and Fig. 2 illustrates anti-LPS IgG levels in two fatal and two non-fatal cases, and also those in a healthy individual. In all sepsis patients, IgG levels (especially IgG against *B. fragilis* LPS) fluctuated over time to a greater extent than levels in a healthy subject. Also fluctuations in IgG against all of the antigens followed similar overall trends, and in most cases anti-bacteroides LPS IgG, especially that against *B. fragilis*, showed greater fluctuations than the anti-enterobacterial/*Pseudomonas* rough LPS IgG. In 5 out of 6 survivors, IgG levels to all of the antigens had increased overall at the end of the sampling period. In 4 of the 6 non-survivors, levels stayed approximately the same or decreased, with an exception showing increasing levels to *B. fragilis* LPS. In 5 out of 6 non-survivors, IgG levels to *B. fragilis* LPS were higher than those against the other LPSs, and in the sixth non-survivor, IgG levels to all antigens were very low throughout the whole sampling period. Three out of 6 survivors and 3 of 6 non-survivors had low initial levels of anti-LPS IgG (i.e. below median levels).

Table 2

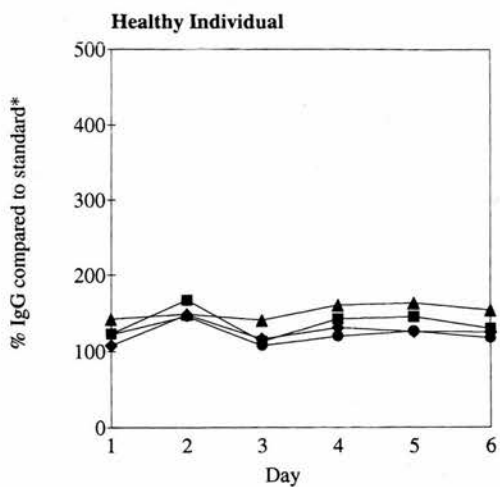
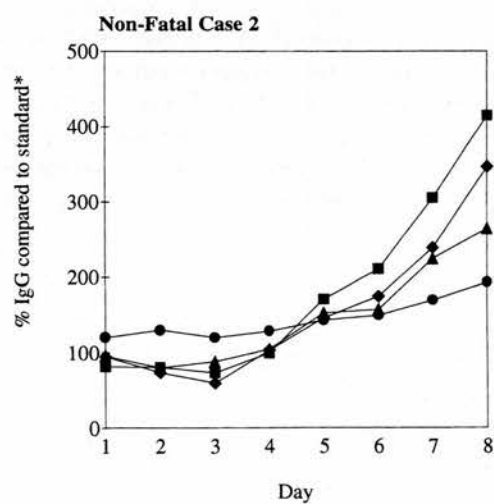
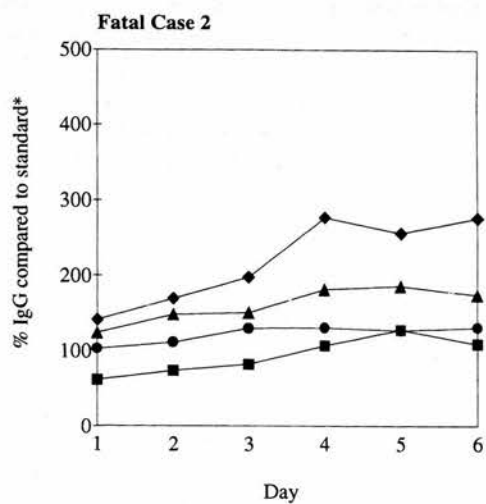
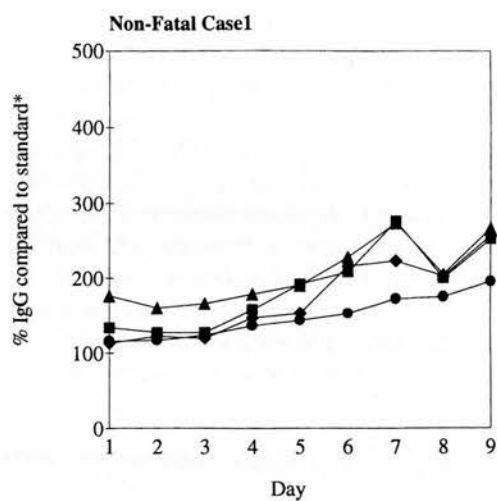
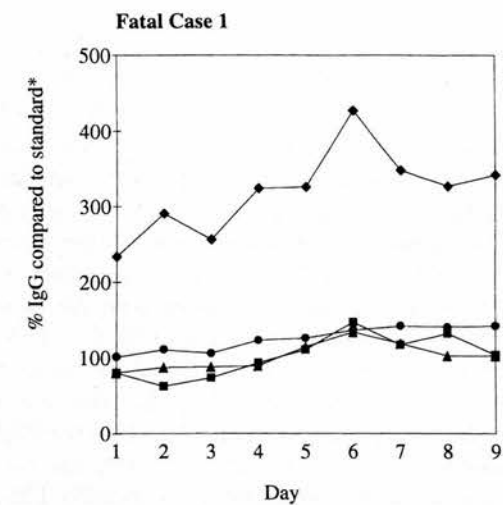
Results of inhibition ELISA measuring cross-reactivity of serum IgG against different LPSs. Figures shown are average percentages calculated from 4 replicates

IgG	Serum pre-incubated with	OD as percent of non-pre-incubated control \pm S.E.
Anti- <i>B. fragilis</i> LPS	<i>B. fragilis</i> LPS	10.1 \pm 4.1
	<i>B. vulgatus</i> LPS	91.5 \pm 1.6
	<i>B. thetaiotaomicron</i> LPS	57.5 \pm 6.6
	Rough LPS mixture	94.2 \pm 4.3
Anti- <i>B. vulgatus</i> LPS	<i>B. fragilis</i> LPS	56.8 \pm 1.1
	<i>B. vulgatus</i> LPS	2.4 \pm 1.1
	<i>B. thetaiotaomicron</i> LPS	21.0 \pm 1.7
	Rough LPS mixture	98.6 \pm 2.7
Anti- <i>B. thetaiotaomicron</i> LPS	<i>B. fragilis</i> LPS	63.9 \pm 1.1
	<i>B. vulgatus</i> LPS	26.2 \pm 0.8
	<i>B. thetaiotaomicron</i> LPS	10.3 \pm 4.1
	Rough LPS mixture	96.4 \pm 2.9
Anti-rough LPS mixture	<i>B. fragilis</i> LPS	89.5 \pm 6.7
	<i>B. vulgatus</i> LPS	98.4 \pm 4.4
	<i>B. thetaiotaomicron</i> LPS	94.2 \pm 3.9
	Rough LPS mixture	12.7 \pm 3.3

OD = Optical density at 405 nm.

S.E. = Standard error.

Rough LPS mixture = *E. coli*, *K. pneumoniae*, *S. typhimurium* and *P. aeruginosa*.



4. Discussion

This study investigated serum levels of IgG against bacteroides LPS in blood donors and in a group of sepsis patients where endotoxin was thought to be derived from the gut. It was decided to look at IgG against aqueous phenol LPS preparations of *B. fragilis*, as this is the most common anaerobic pathogen, and also *B. vulgatus* and *B. thetaiotaomicron*, as these are two of the most numerate of the *Bacteroides* spp. in faeces [22]. Since enterobacteria and closely related species are thought to be responsible for the development of sepsis, we also measured IgG against a mixture of rough LPS from three enterobacteria plus *P. aeruginosa*. It is important to stress that the aim of this study was not to make direct comparisons between bacteroides LPS and the rough LPS mixture, but rather to observe and compare the IgG responses in order to see whether that against bacteroides LPS was significant. It is also important to emphasise that the aqueous phenol extracts of bacteroides were not pure LPS, but also contained some capsular polysaccharide and protein (E. Allan, unpublished data). However, it was decided not to try to purify the LPS further, as we were trying to mimic the in vivo situation as closely as possible; if hot phenol failed to separate the surface components of bacteroides completely, then the LPS is unlikely to be present in pure form in the body.

In the blood donor population, similar distributions of IgG levels were observed for all antigens tested. It has previously been suggested that IgG levels against enterobacterial LPS core are dependent on the degree of long-term exposure, which in turn may be dependent on the amount of LPS which translocates naturally across the gut wall and into the circulation [11]. Our results suggest that this could also be true for bacteroides LPS.

In all sepsis patients, IgG levels against all antigens followed similar overall trends. Inhibition ELISAs showed that whilst there were some epitopes

shared between the different bacteroides LPSs, there were very few (if any) between bacteroides LPS and the enterobacterial/*Pseudomonas* LPS mixture. This is not surprising given that the lipid A structure of *B. fragilis*, and probably the other *Bacteroides* spp. is markedly different from that of the enterobacteria [16,23]. In some cases, a sudden consumption of antibody was shown to be concurrent with an episode of endotoxaemia (confirmed previously by LAL assays (Sepsis Intervention Group, unpublished data)). In other cases, there was a decrease in IgG against all antigens but no endotoxin detected in the serum. However, endotoxaemia is often very transient and may not be detected in a LAL assay [24], so IgG levels may be a more accurate method for detecting it. The ELISA used in this study appears to be a good method of detecting an acute episode of endotoxaemia indirectly.

One of the most striking observations in this study was that in 5 out of 6 non-survivors, IgG levels against *B. fragilis* LPS were higher than levels of IgG against the other antigens. This only occurred in one survivor. Due to the small number of sepsis patients studied here, it is not possible to draw any firm conclusions linking certain antibody levels or kinetics to a fatal or non-fatal outcome. Moreover, by the time the first blood sample is taken, the patient is often very sick, and the antibody levels at that time may bear no resemblance to those in health. However, the study has demonstrated a definite IgG response to bacteroides LPS which is independent of, but shows similar overall trends to, the response to enterobacterial LPS. Other recent studies in our laboratory have shown bacteroides LPS to be more biologically active in certain endotoxin assays than previously reported [25]. This, together with the nature of the IgG response, suggests that bacteroides LPS could be more important than previously thought in the development and outcome of sepsis.

In summary, we have demonstrated for the first time that healthy adults have individual endogenous

Fig. 2. (Opposite) Kinetics of anti-LPS IgG response in 4 sepsis patients (2 survivors, 2 non-survivors) and in a healthy individual over 6- to 9-day periods. Serum from a donor expressing close to median levels of IgG to all LPSs tested, selected from a screen of 641 random blood donors, was included on all plates to standardise OD readings. ♦, *B. fragilis* NCTC 9343; ■, *B. thetaiotaomicron* NCTC 10582; ▲, *B. vulgatus* MPRL 1985; ●, Enterobacterial/*Pseudomonas* rough LPS mixture. Points shown are average percentages calculated from at least 4 replicates. * Standard = 100%.

levels of IgG against bacteroides LPS. We have also shown that the anti-bacteroides LPS IgG is not simply anti-enterobacterial LPS IgG which cross-reacts with bacteroides LPS, but that the two are independent of each other. We have also demonstrated certain trends in anti-bacteroides LPS IgG kinetics in sepsis patients which suggest that bacteroides LPS could be significant in sepsis and indicate that the study should be extended.

Acknowledgements

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